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Phylogenetic placement and population genetic analysis of the endangered winged mapleleaf, Quadrula fragosa

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Phylogenetic placement and population genetic analysis of the endangered winged mapleleaf, *Quadrula fragosa*

by

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TABLE OF CONTENTS

LIST OF FIGURES

Boostrapping was used to account for the differences in sample sizes in each population, and values are based on 999 replications and displayed above the branches.

LIST OF TABLES

CHAPTER 1 - INTRODUCTION

The freshwater mussel, *Quadrula fragosa* (Conrad 1835), is one of the many imperiled freshwater bivalves in North America. Even though the family Unionidae reaches its greatest diversity in North America, this family of freshwater mussels is the most endangered group of animals on the continent (Graf and Cummings, 2007; Williams *et al.,* 1993). Williams *et al.* (1993) reported that 43% of the 297 species and subspecies of freshwater mussels in North America were threatened, endangered, candidates for federal listing, or extinct. The winged mapleleaf, *Quadrula fragosa,* is one such species. Reductions in the range of *Q. fragosa* to a single population in the St. Croix River between Minnesota and Wisconsin (USFWS, 1991) ultimately led to the listing of this species as endangered.

Range reduction has be so severe that populations in the type locality of *Quadrula fragosa*, the Scioto River in Ohio (Conrad, 1835), have been extirpated. Conrad (1835) described the species as having a similar morphology to *Quadrula quadrula* (Rafinesque, 1820), but "is much more ventricose, has more prominent tubercles, and is very distinct." Though Conrad (1835) described the species as being distinct, the large amount of shell variation in the *Quadrula* genus does not help with morphological and taxonomic confusion between *Q. fragosa* and *Q. quadrula.* Historically, *Quadrula fragosa* was found at a low frequency in Mississippi River drainages such as the Tennessee, Ohio, and Cumberland River drainages among ten US states, including Ohio, Indiana, Kentucky, Tennessee, Illinois, Wisconsin, Iowa, Missouri, Nebraska, and Oklahoma (USFWS, 1991) (Fig. 1). Due to habitat degradation, pollution, and river channel modifications, the species was thought to be

reduced to a single population in the St. Croix River between Minnesota and Wisconsin, a 99% reduction from their historical range (Fig. 2) (USFWS, 1991). Because of this reduction in range, the United States Fish and Wildlife Service (USFWS) listed *Quadrula fragosa* as federally endangered in 1991 under the Endangered Species Act of 1973 (USFWS, 1991). Since the time of its listing *Q. fragosa* morphotypes have been found in locations outside of the St. Croix River including the Bourbeuse River in Missouri (Andy Roberts, pers. com.), the Saline and Ouachita Rivers in Arkansas (Posey *et al.,* 1996), and the Little River in Oklahoma (Caryn Vaughn, pers. com.). It has been speculated that populations of *Q. fragosa* were historically found in the Kiamichi River River in Oklahoma, but records are uncertain.

It is difficult to fully examine a species like *Quadrula fragosa* when little is known about the natural history traits. For example, some studies (Hornbach *et al.*, 1996; Steingraeber *et al.,* 2006) have been done to examine the habitat of the mussel, but little is known about its internal anatomy. Like other mussels, *Q. fragosa* is a filter feeder that feeds on diatoms, algae, protozoans, and other microscopic debris in the water (Davidson, 1997). *Quadrula fragosa* embed in the substrate of a stream or river, which usually consists of firm sand or gravel to large cobble with silt (Harris, 2006). Harris (2006) described the water velocity for the habitat of *Quadrula fragosa* as slow-moving to fairly swift currents in Arkansas, and Hornbach *et al.* (1996) calculated an average water velocity of 0.19 m/sec, and an average depth to the mussel bed as 0.98 meter in the Minnesota/Wisconsin. The stability of the substrate and respective habitat has a larger impact on a mussel community than the particle size of the substrate (Hornbach *et al.*, 1996). It is important to note that the depth at which a mussel is burrowed can have a large impact on mussel communities; a water level that is too low can lead to high mussel community mortality (Hornbach *et al.*, 1996). Due to

river modifications, the large free-flowing river habitat with many side channels and flood plains has been altered by the addition of dams and reservoirs, fragmenting the previous habitat and decreasing the floodplain areas (USACE, 2007).

Most unionids are dioecious and females can produce up to 500,000 eggs (Oesch, 1984). Sperm is released into the water where it is taken up by the female via the incurrent siphon (Vaughan, 1997). Zygotes of *Quadrula fragosa* develop into parasitic glochidia, and are released from the female into the water via the excurrent siphon where the passively infect a specific host fish (Oesch, 1984). The brooding period for *Q. fragosa* is from late May through mid-July (Vaughan, 1997), and lasts approximately five weeks (Heath *et al.*, 2000). Studies by Hove *et al.* (in review) and Steingraeber *et al.* (2006) observed varying rates of metamorphosis from glochidia to juvenile with temperature. Hove *et al.* (in review) observed that an increase in water temperature resulted in an increased rate in development during metamorphosis. Juveniles excysted from their host fish when the daily average water temperature was between 17 and 20°C (Steingraeber *et al.,* 2006). *Quadrula fragosa* take less time to complete their metamorphosis in the spring, and excyst earlier than other mussel species, possibly giving an advantage in resource competition during the first year of growth (Steingraeber *et al.*, 2006).

Two species of catfish, the channel catfish (*Ictalurus punctatus)* and the blue catfish (*Ictalurus furcatus)* are thought to be the host fish for *Quadrula fragosa* (Hove, 2004; Steingraeber *et al.,* 2006). The blue catfish is broadly distributed in rivers along the Gulf of Mexico, becoming restricted to the Mississippi and Missouri Rivers in the north (Lee, 1980). The blue and channel catfish usually spawn in June, with frequent occurrences throughout the summer (Smith, 1979), coinciding with the brooding season of *Q. fragosa*. This is

important because *Q. fragosa* requires a host fish to help in the offspring's metamorphosis to adults (Oesch, 1984). Due to the construction of dams, the upstream migration of blue catfish is inhibited, limiting its northern abundance (Smith, 1979). The channel catfish has a much more extensive distribution, covering the middle of the United States from Florida and New Mexico to Montana and New York (Lee, 1980). Both catfish prefer clear, medium to large rivers with a swift current (Lee, 1980), similar to the habitat of *Q. fragosa.* While juveniles metamorphosed on both species of catfish, the channel catfish may be a more preferred host for the St. Croix population because it is more frequently found in northern sites year-round, where the blue catfish is not usually found (Steingraeber *et al.*, 2006).

Anthropogenic disturbances have affected river channels as well, including recreational and commercial boating (Vaughan, 1997) and damming of rivers. These disturbances have caused a substantial decrease in mussel population in the 1980's (Vaughan, 1997), and making the mussels more vulnerable to chance and accidental events, such as low water, chemical spills, and climate changes (Vaughan, 1997). Twenty-seven dams maintained a 2.75-meter deep channel in the Mississippi River so that it would be navigable for commercial boating (Smart *et al.*, 1986) (Fig. 3). The first dam was completed in 1917, and the series of dams now stretch from Minneapolis, Minnesota to St. Louis, Missouri (USACE, 2007). In 1929, a similar channelization project was completed by the United States Army Corps of Engineers on the Ohio River (USACE, 2007). Channelization, similar to dam introduction, increases channel siltation through soil erosion, increases the temperature of the river, and decreases the water table (Davidson, 1997), all of which can have a detrimental effect on mussel populations.

Dams can also negatively affect fish populations by obstructing migrations to spawning and feeding areas (Northcote, 1998). Paddlefish passage through dams occurred mostly in the spring and corresponds to migrations for spawning (Zigler, 2004). Because of strong water velocities, paddlefish movements were more affected moving upstream than downstream (Zigler, 2004). This hindered movement could result in a decrease of spawning success, due to decreased spawning success or the inability of recruits to return to spawning grounds (Zigler, 2004). Whether channel catfish have the same difficulty of dam navigation needs to be investigated to determine whether it may have subsequent affects on *Q. fragosa* distribution. Hove *et al.* (in review) concluded that the population decline in *Q. fragosa* is not due to host fish decline, because the channel catfish is still relatively abundant in the St. Croix River, but we are unaware of how the catfish population has changed in other localities where *Q. fragosa* previously or currently inhabit.

An additional historical pressure to freshwater mussel species was the button and pearl industries. The button industry began in the 1800's, and peaked in the early 1900's, when it was discovered that the attractive and sturdy nacre on the inside of freshwater mussel shells could be used for buttons (Nedeau *et al*., 2000). A commercial fishery arose to keep up with demand, and it was found that the best shells were from rivers in the Midwest because of their thickness (Nedeau *et al.*, 2000). The high demand for pearl buttons had a detrimental effect on the native freshwater mussel populations. The invention of plastic caused the pearl-button industry to cease in the mid-1900's because plastic was easier and cheaper to manufacture (Nedeau *et al.*, 2000), taking the industrial demands off of the mussel species.

The pearl industry began in the 1900's when the Japanese found that the freshwater mussel shell's nacre would form layers over an artificially placed bead to produce a pearl (Nedeau *et al.*, 2000). The cultured pearl industry became prevalent in Japan, China, Indonesia, French Polynesia, and Australia (Nedeau *et al.*, 2000). In the 1980's, the United States exported more than 25,000 tons of freshwater mussel shells, but because of decreasing stocks and harvest restrictions, exports fell to less than 10,000 tons (Nedeau *et al*., 2000). Both of these industries have had detrimental effects on mussels due to over harvesting in the past.

The industrial history, habitat changes, morphological confusion, and endangered of *Quadrula fragosa* make it an interesting species to study. This study will look at different angles to understand the species, its population dynamics, and suggestions for the preservation of the species. My thesis project has two components. First, I will determine the phylogenetic placement of *Q. fragosa* within the genus *Quadrula,* and verify that individuals from the southern populations in Missouri, Arkansas, and Oklahoma that contain animals that are morphologically similar to *Q. fragosa* are genetically *Q. fragosa.* Phylogenetic relationships based on genetic data are important to resolve the ambiguity based on morphological characters within the *Quadrula* genus. This component of the study will verify the listing as an endangered species by the USFWS in 1991, and determine the full extent of the distribution of *Q. fragosa.* The results of the phylogenetic study will be vital for the USFWS to begin developing conservation strategies based on the within and between population dynamics. Second, I will determine the amount of genetic diversity and genetic differentiation within and among populations of *Quadrula fragosa* through the development and implementation of microsatellite markers. Microsatellites, also called short sequence

repeats (SSR) or short tandem repeats (STR), are made up of 1-6 nucleotides tandemly repeated (Toth *et. al,* 2000). They can be found in non-coding and coding regions of DNA (Toth *et. al,* 2000), and display a high mutation rate (Jarne and Lagoda, 1996). High variability, due to the high mutation rate, makes microsatellites valuable for increasing the available statistical power to determine genetic differentiation (Hedrick, 2001). This component will utilize various analyses to determine how genetic variation is distributed and determine if any evidence of gene flow exists between populations of *Q. fragosa.*

This thesis includes a description and conclusions from the phylogenetic study, a report on the development of the twelve microsatellites, and a discussion about the application of these microsatellites to better understand the population genetics of *Q. fragosa* populations. Results from this thesis will be forwarded to the United States Fish and Wildlife Service and the United States Army Corps of Engineers to aid in future conservation decisions and management programs. It will also provide insight into microsatellite development and application, and serve as a stepping-stone into the understanding of other endangered populations of freshwater mussels to quickly assess and take action to prevent extinction.

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Figure Legends

Figure 1. Historical distribution of *Quadrula fragosa* within the United States of America. *Quadrula fragosa* was historically found in the states Ohio (OH), Indiana (IN), Kentucky (KY), Tennessee (TN), Wisconsin (WI), Illinois (IL), Minnesota (MN), Iowa (IA), Missouri (MO), Arkansas (AR), and Louisiana (LA), and in the Mississippi, Ohio, Cumberland, and Tennessee River (R.) drainages. It has been speculated that *Q. fragosa* was historically found in the Kiamichi River, Oklahoma, but records are uncertain.

Figure 2. Present distribution of *Quadrula fragosa* within the United States of America. Populations from the current distribution are found in the St. Croix River between Minnesota (MN) and Wisconsin (WI), Bourbeuse River, Missouri (MO), Saline River, Arkansas (AR), Ouachita River, Arkansas (AR), and Little River, Oklahoma (OK).

Figure 3. Map of populations, river systems, and location of dams. The upstream and downstream limits of the dams are indicated with hash-marks. There are 29 dams on the Mississippi River between St. Paul, Minnesota and St. Louis, Missouri, one on the Saline River, Arkansas, approximately seven on the Ouachita River, Arkansas, and two on the Little River in Oklahoma.

Figure 1.

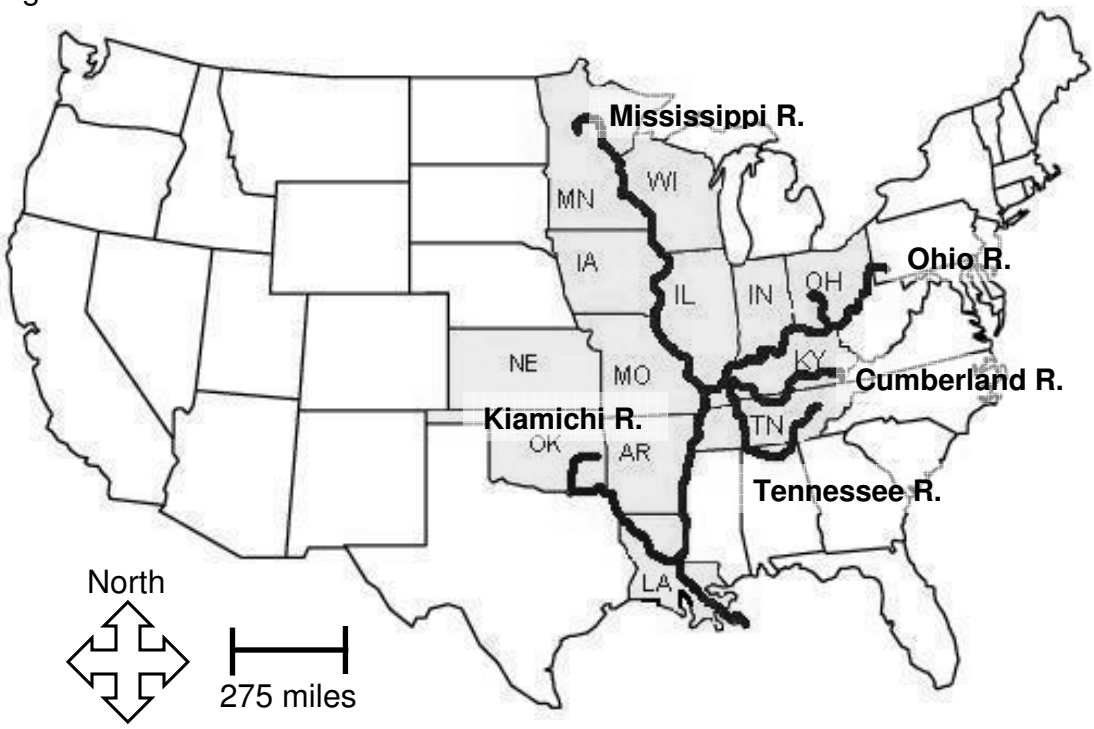
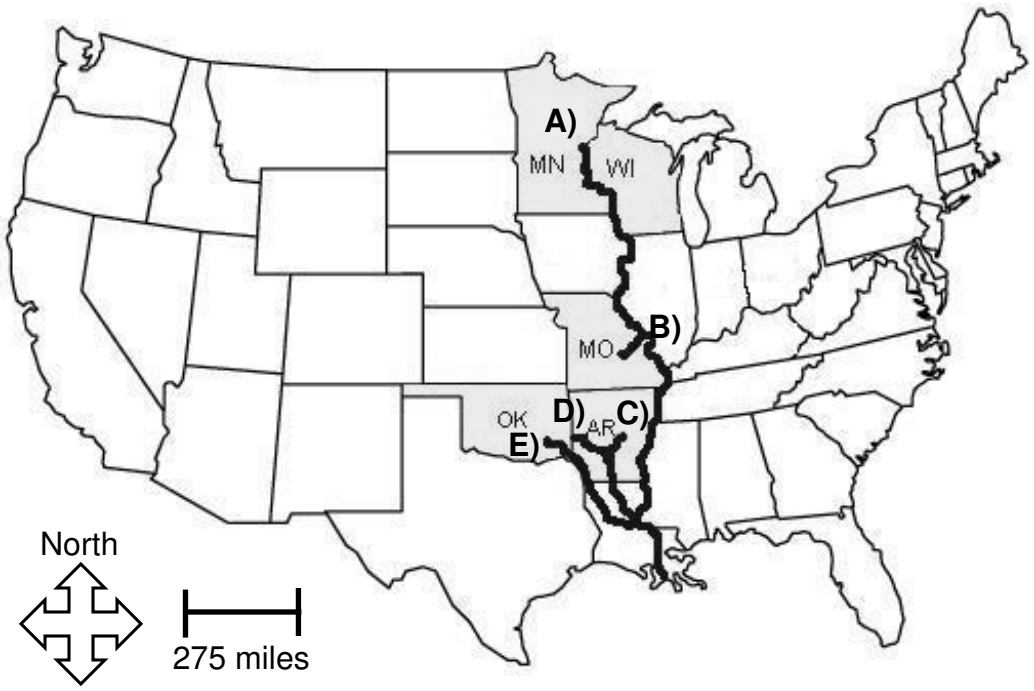
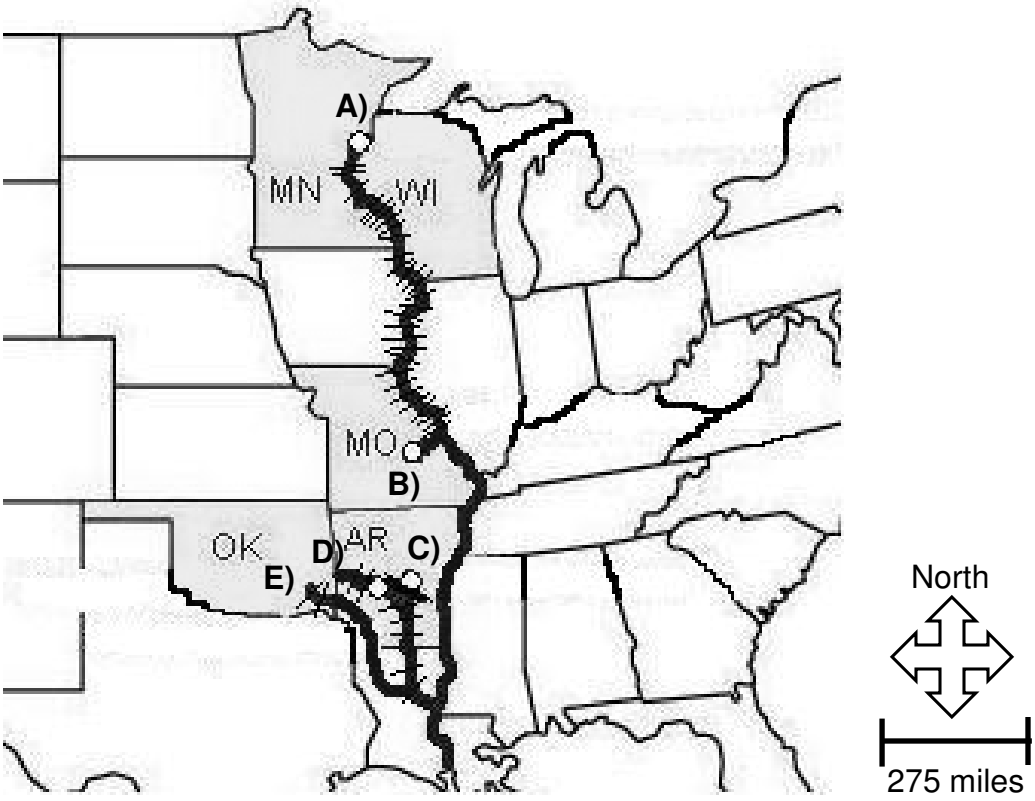

Mississippi R.
Ohio R.
Cumberland R.
Kiamichi R.
Tennessee R.
MN
WI
IA
IL
IN
OH
NE
MO
KY
TN
OK
AR
LA
North
275 miles

Figure 2.



Legend:
A) St. Croix River, Minnesota/Wisconsin
B) Bourbeuse River, Missouri
C) Saline River, Arkansas
D) Ouachita River, Arkansas
E) Little River, Oklahoma

Figure 3.



Legend:
A) St. Croix River, Minnesota/Wisconsin
B) Bourbeuse River, Missouri
C) Saline River, Arkansas
D) Ouachita River, Arkansas
E) Little River, Oklahoma

CHAPTER 2 - PHYLOGENETIC PLACEMENT OF *QUADRULA FRAGOSA* WITHIN THE GENUS *QUADRULA* (UNIONIDAE) USING MITOCHONDRIAL ND1 SEQUENCES

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ABSTRACT

The endangered winged mapleleaf, *Quadrula fragosa* once occurred in the Mississippi, Tennessee, Ohio, and Cumberland River basins in the United States. While the range of this species was thought to have been reduced to a single population in the St. Croix River (Minnesota/Wisconsin), individuals morphologically similar to *Q. fragosa* recently have been recorded in three southern states: Missouri, Arkansas, and Oklahoma. The aim of this study was two-fold. First to determine if *Q. fragosa* is a distinct species from *Q. quadrula.* Second, to determine whether these new records represent from the southern states represent true *Q. fragosa.* To address these aims, we employed a phylogenetic analysis of DNA sequences from the mitochondrial ND1 genes. Sequences for the nuclear Histone3 gene region were explored, but due to the high degree of conservation, phylogenetic analysis was not pursued further. Our results indicate that *Q. fragosa* is a separate species from *Q. quadrula* and that the southern populations are genetically *Q. fragosa*. The results of this study have identified a number of new disjunct populations of *Q. fragosa*. Considering the endangered status of this species, future conservation work should address genetic variation within and among the populations as well as the amount of gene flow between populations. Only then will the populations be rehabilitated, expanded, and removed from the endangered species list.

INTRODUCTION

Members of the bivalve family Unionidae, Fleming 1828, can be found on every continent in the world, except Antarctica, and reach their greatest diversity in the rivers and streams of North America (Graf and Cummings, 2007). The freshwater bivalves of North America have been declining (Bogan, 1993), and, consequently, are the most endangered group of animals in North America (Williams *et al.,* 1993).

One such endangered mussel species is *Quadrula fragosa* (Conrad 1835). *Quadrula fragosa* belongs to the diverse genus *Quadrula* (Rafinesque 1820), comprised of 20 different species with five listed as federally endangered and three presumed extinct (Williams *et al.,* 1993). Members of *Quadrula* are characterized by a high degree of shell phenotypic plasticity. However, traditional taxonomy of *Quadrula* is based on shell morphology and characteristics (e.g. Williams and Mulvey, 1994). Therefore, the routine identification of individual specimens based solely on shell morphology can be difficult (Williams and Mulvey, 1994).

Originally described by Conrad in 1835, the taxonomy of *Quadrula fragosa* has been confusing. The species was described as distinct by Conrad in 1835, but then synonomized with *Quadrula quadrula* by Isely in 1925 (Isley 1925). This designation was consistent with other authors (Isley 1925; Neel 1941; Burch 1975). Clinal variation, intergrades in shell sculpture (e.g. rows of tubercles, ridges, area between valves) of individuals in river systems, has been observed in unionid bivalves (Ortmann, 1920) including several species of *Quadrula* (i.e. *Q. cylindrical, Q. metanevra, Q. pustulosa*). For example, individuals found upstream are usually thinner with a smoother shell shape, becoming fatter and having more tubercles moving downstream (Ortmann, 1920). If one were to see an individual at the

beginning of a river and one at the mouth of a river, it might be mistakenly identified as two different species. Unionid taxonomic expert, Dr. David H. Stansbery (Ohio State University Museum) argued that *Q. fragosa* was a distinct species stating that "there were no known intergrades between *Q. fragosa* and other members of the *Quadrula* complex" based on morphological characters (Vaughan, 1997). The taxonomic status of *Q. fragosa* became important to the USFWS because people working in the field need to know whether an individual mussel is the endangered *Q. fragosa* or the non-endangered *Q. quadrula.*

The United States Fish and Wildlife Service (USFWS) listed Quadrula fragosa as federally endangered on June 20, 1991 under the Endangered Species Act of 1973 (USFWS, 2005). At the time of listing, it was believed that *Q. fragosa* was reduced to a single population in the St. Croix River between Minnesota and Wisconsin (Vaughan, 1997). However, the historical distribution of the species was much wider. Populations of *Q. fragosa* were recorded in ten states across the US, in the Mississippi, Tennessee, Ohio, and Cumberland River drainages (USFWS, 1991). Since the federal listing of *Q. fragosa* as an endangered species, morphotypes of *Q. fragosa* have been found in rivers in Missouri (Andy Roberts, pers. com.), Arkansas (Posey *et al.,* 1996), and Oklahoma (Caryn Vaughn, pers. Com.) (Fig. 1). We are unsure if the morphotypes are genetically *Q. fragosa,* or the closely related species, *Q. quadrula.* If they are genetically *Q. fragosa,* they will certainly need to be incorporated into conservation management plans.

The aims of this study are two-fold. First, was to determine the validity of the species *Q. fragosa* and its placement within the genus *Quadrula* using mitochondrial (NADH dehydrogenase, ND1) and nuclear (Histone H3) DNA sequences in a phylogenetic analysis. Second, this study will determine whether the new southern populations are genetically *Q.*

fragosa using the gene regions mentioned above in a phylogenetic analysis. This datum will clarify some of the morphometric ambiguity, and help conservation agencies develop appropriate management and rehabilitation strategies. These data will be used to determine the current range of *Q. fragosa* and will be applied to the management and continued conservation of the species.

MATERIALS AND METHODS

Taxa sampled

A total of 37 individuals of *Quadrula fragosa* from five locations were sampled for ND1 sequences (Table 1) in this study, and 34 individuals from five locations and six species were sampled for Histone H3 sequences. For ND1, twelve individuals are from the St. Croix River, Minnesota/Wisconsin and were treated as the genetically "known" population of *Q. fragosa*. In addition, four southern populations with morphologically identified "*Q. fragosa*" individuals were sampled: three individuals from the Bourbeuse River, Missouri, ten individuals from the Saline River, Arkansas, nine individuals from the Ouachita River, Arkansas, and three individuals from the Little River, Oklahoma (Fig. 2). These sequences were added to a subset of 20 species/ 36 specimens from Serb *et al.* (2003). The Histone H3 dataset consisted of eight individuals from the St. Croix River, Minnesota/Wisconsin, three individuals from the Bourbeuse River, Missouri, four individuals from the Saline River, Arkansas, and three individuals from the Little River, Oklahoma were sampled. As a comparison, four other species of *Quadrula* were sequenced for Histone H3: *Q. quadrula* (n=6), *Q. pustulosa* (n=2), *Q. metanevra* (n=2), *Q. rumphiana* (n=3), and *Q. apiculata* (n=3). All tissues were collected by collaborators and stored in 95% ethanol. Total genomic DNA

was extracted from mantle tissues and isolated using the spin-column protocol for animal tissues of the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, California). DNA integrity was determined using a 1% agarose gel, and concentration was determined with UV fluorescence using NanoDrop, model ND-1000.

PCR Amplification and Sequencing

ND1

The mitochondrial ND1 (NADH dehydrogenase) gene was amplified using the primers Leu-uurF (5'-TGG CAG AAA AGT GCA TCA GAT TAA AGC-3') and LoGlyR (5'-CCT GCT TGG AAG GCA AGT GTA CT-3') (Serb *et al.,* 2003). The PCR reaction mix consisted of a 25 ul reaction with: 16.25 ul of water, 2.5 ul of 10X buffer, 2.5 ul of 25mM $MgCl_2$, 1 ul of 10 uM each primer, 0.5 ul of 10mM dNTPs, 0.25 ul *Taq* polymerase, and 1 ul of 20-30 ng/ul DNA. The thermocycler program was 96°C for 3 min; 35 cycles of 96°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and a final extension of 72°C for 3 minutes. PCR products were visualized in a 1% agarose gel and SYBR-safe DNA gel stain. Products that displayed the desired band size were cleaned using an Exo-Sap® (US Biochemical) reaction according to manufacturer's instructions.

The sequencing reaction used the Applied Biosystem's PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1. The 10 ul reaction was as follows: 2 ul Big Dye, 2 ul of 5X buffer, 1 ul of 10 μM forward or reverse primer, 1-2 ul Exo-sapped PCR product, and was brought to 10 ul using water. Thermocycler conditions were: 96°C for one minute; 35 cycles of 96°C for 10 seconds, 55°C for 5 seconds, and 60°C for 4 minutes. Following an ethanol/EDTA clean-up according to the manufacturer's instructions, both the

forward and reverse strands were sequenced at the Iowa State University DNA Facility on an Applied Biosystems 3730xl DNA Analyzer. Sequences were edited and by eye aligned using BioEdit (Hall, 1999).

Histone H3

The nuclear Histone H3 gene region was amplified using the primers H3altF (5'-ATG GCT CGT ACC AAG CAG AC-3') and H3R (5'-ATA TCC TTR GGC ATR ATR GTG AC-3') (Colgan *et al.,* 1998). The PCR and thermocycler conditions were the same as above, with the exception of an annealing temperature at 54°C. PCR product visualization, clean-up, and sequencing were performed using the same protocol as described above.

Maximum Parsimony Analysis

Sequences were aligned using BioEdit (Hall, 1999). The analysis in PAUP* 4.08b (Swofford, 2002) used maximum parsimony (MP) to infer phylogenetic relationships. The analysis was performed using 100 addition replicates of a heuristic search with ACCTRAN, MULPARS, and TBR branch-swapping options. Only minimum-length trees were saved, and branches with a length of zero were collapsed. All characters had equal weight and treated as unordered for the analysis. Indices of branch support for the individual nodes were calculated via bootstrapping using the FAST stepwise addition option. The outgroups, *Megalonaias nervosa, Amblema plicata,* and *Fusconaia flava* were based on Serb *et al.* (2003) results.

The Phylogenetic Species Concept (PSC) is historically based and includes the criterion of monophyly in the general sense (de Quieroz and Donoghue, 1988) or

"exclusivity," where an exclusive group of organisms is one whose members are more closely related to each other than they are to any organisms outside the group (Baum and Donoghue, 1995). The PSC was applied to our study to determine species groups.

Bayesian Analysis

Partitioned Bayesian phylogenetic analyses were conducted using MrBayes v. 3.0b4 (Ronquist and Huelsenbeck, 2003). Given the heterogeneity of DNA sequence models of evolution within and/or across genes (e.g. Nylander, 2004; Brandley *et al.,* 2005); the mtDNA data were divided into three different partitions for each codon position of the protein coding ND1 gene. Appropriate models of sequence evolution for each partition were determined using Akaike Information Criterion (AIC) implemented with MrModeltest v. 2.2 (Nylander, 2004). The same outgroups for the maximum parsimony analysis were used for this analysis. Two simultaneous, completely independent partitioned analyses were run to help determine when convergence had been achieved (i.e., stationarity). The analysis was run for six million generations, with a random starting tree, and four incrementally heated Markov chains (using default heating values), sampling the Markov chains at intervals of 100 generations (25,000 generations discarded as burn-in). Clades with posterior probabilities of greater-or-equal than 0.95 were considered strongly supported (Wilcox *et al.,* 2002; Alfaro *et al.,* 2003).

Genetic Distance

Pairwise genetic distances with the ND1 gene were calculated across a subset taxa comparing all individuals within *Quadrula fragosa,* all individuals within *Q. quadrula, Q.*

fragosa vs. *Q. quadrula, Q. fragosa* vs. the *quadrula* species group, *Q. fragosa* vs. the *pustulosa* species group, and *Q. fragosa* vs. the *metanevra* species group using the program PAUP* 4.08b (Swofford, 2002). The model of sequence evolution (GTR+I+G) was determined from the data in the program MrModeltest (Nylander, 2004). The calculations were used to compare genetic distances within *Quadrula fragosa* and among other *Quadrula* species.

RESULTS

Out of 34 individuals of *Quadrula fragosa* sequenced for the Histone H3 gene region, all base pairs except five were conserved across the following six species, *Q. metanevra, Q. pustulosa, Q. apiculata, Q. rumphiana, Q. quadrula,* and *Q. fragosa.* Four of these base pair differences were observed in *Q. metanevra* and *Q. pustulosa.* However, at base pair 250, *Q. metanevra, Q. pustulosa,* and most of the sampled *Q. fragosa* contained a G while *Q. quadrula, Q. apiculata, Q. rumphiana,* and two *Q. fragosa* individuals from the Saline River, Arkansas contained a C. This base change is in the third codon position, and did not change the amino acid. Phylogenetic analysis was not pursued due to the high degree of conservation of this gene region.

Even though 37 individuals were sampled for ND1 DNA sequences, a subset consisting of at least two individuals from each *Q. fragosa* population was utilized for the phylogenetic analysis (12 specimens total). Eight unique haplotypes were found across individuals, exhibiting the low amount of variation in *Quadrula fragosa.*

Maximum Parsimony Analysis

Out of 595 total characters used for the mitochondrial ND1 gene, 349 were constant and 207 were phylogenetically informative under MP. The analysis resulted in a 216 most parsimonious trees (MPT) of tree length (TL) 658 (CI = 0.488, RI = 0.826). Figure 3 shows an example of one of the maximus parisimonious trees, and figure 4 displays the strict consensus tree from the maximum parsimony analysis. *Quadrula fragosa* samples from the St. Croix and *fragosa*-morphotypes from the southern populations form a well-supported clade (bootstrap = 100). The bootstrap value of 100 supports the distinctiveness of *Q. fragosa* from the other members of the *quadrula* species group (*Q. apiculata, Q. rumphiana,* and *Q. quadrula*) (Fig. 4). A clade containing *Q. quadrula, Q. apiculata*, and *Q. rumphiana* is resolved as a sister group to *Q. fragosa.* However, this relationship is not well supported because the bootstrap value is less than 50 (Fig. 4).

Bayesian Analysis

Akaike information criterion indicated that the best fit model of sequence evolution for the ND1 gene was GTR+I+G (General Time Reversal + Invariant site + Gamma distribution of rate). The two independent partitioned analyses converged on similar average log-likelihood values (-lnL=3501.04). A 50% majority rule consensus tree of the combined pool of trees (57,500 trees) was applied. Figure 5 displays the results of the Bayesian analysis. As in the MP analysis, all *Q. fragosa* specimens from the five localities formed a monophyletic clade, supported with a posterior probability (PP) of 100. However, unlike the MP analysis, the *quadrula* species group is not monophyletic, as *Q. fragosa* is not the sister group to the *quadrula* species group. Instead, *Q. fragosa* is recovered as the sister to the *Q.*

metanevra species group. This relationship is supported by a posterior probability of 67 (Fig. 5).

Genetic Distance

Genetic distance was calculated using the same model of evolution used in the maximum parsimony and Bayesian analyses, GTR+I+G (General Time Reversal + Invariant site + Gamma distribution of rate). Sequence divergence among individuals of the *Q. fragosa* ranged from 0 to 0.37% (average = 0.06%; Table 2). Individuals within the species *Q. quadrula* ranged from 0 to 7.04% (average = 0.32%; Table 2). Sequence divergence of *Q. fragosa* vs. *Q. quadrula* ranged from 12.24% to 13.12% (average = 12.54%; Table 2), and sequence divergence of *Q. fragosa* vs. the remainder of the *quadrula* species group (*Q. quadrula, Q. apiculata,* and *Q. rumphiana*) ranged from 12.24% to 16.19% (average = 13.19%; Table 2). Finally, sequence divergence of *Q. fragosa* vs. the *metanevra* species group ranged from 16.25% to 21.04% (average = 18.27%; Table 2).

DISCUSSION

Utilizing ND1 gene sequences, we determined that *Q. fragosa* is a separate species from *Q. quadrula,* due to the distinct monophyletic clades formed by *Q. fragosa* and *Q. quadrula.* The validity of *Q. fragosa* as a species was shown by employing the PSC (Mishler and Brandon, 1987; de Queiroz and Donoghue, 1988, 1990; Mayden, 1977). The PSC is historically based and includes the criterion of monophyly in the general sense (de Quieroz and Donoghue, 1988) or "exclusivity," where an exclusive group of organisms is one whose members are more closely related to each other than they are to any organisms outside the

group (Baum and Donoghue, 1995). This conclusion justifies the endangered listing by USFWS (1991) and directs conservation managers to examine population structure in developing appropriate plans for the preservation of the species.

The second component of this study was to determine if the southern "morphotypes" were true *Q. fragosa*. Congruent results of the MP and Bayesian analyses resolved all sampled individuals of *Q. fragosa* into a well-supported monophyletic clade, distinct from other species of *Quadrula*. After the dramatic range reduction during the 1900's, the last remaining population of *Q. fragosa* was believed to be in the northern St. Croix River between Minnesota and Wisconsin. Our study shows that the range of *Q. fragosa* is larger than described at its listing, expanding the species range outside of the historical range into a new river in Missouri, and a new state, Arkansas. We anticipate the discovery of additional populations in adjacent river systems as mussel communities are censused.

While the MP analysis resolved *Q. fragosa* within the same clade as *Q. quadrula, Q. apiculata,* and *Q. rumphiana,* the Bayesian analysis resolved *Q. fragosa* as a sister group to the *metanevra* species group and not with the *quadrula* species group. This is encouraging for identifying *Q. fragosa* as a species, but shows the ambiguity with *Q. fragosa* sister group relationships. The MP analysis identifies the remainder of the *quadrula* species group (*Q. quadrula, Q. apiculata,* and *Q. rumphiana*) as sister to *Q. fragosa*, but the Bayesian analysis identifies the entire *metanevra* species group as sister to *Q. fragosa*. However, neither of these relationships was well supported with posterior probabilities or bootstrap support. Contrasting results is most likely due to using a parsimonious approach vs. a mathematical evolution model for the phylogenetic analyses. An important goal for the USFWS was to

resolve uncertainties with the relationship of *Q. fragosa* and *Q. quadrula,* and since both analyses identify *Q. fragosa* as its own species, this study has satisfied said goal.

When looking at genetic divergences of *Quadrula fragosa,* the range of divergence within the species is quite lower than the range for individuals within *Q. quadrula.* The amount of genetic divergence is more exemplified when comparing *Q. fragosa* to the *Q. quadrula* species and other *Quadrula* species groups. For example, individuals within *Q. fragosa* had approximately five times less the amount of genetic divergence compared with individuals within *Q. quadrula.* When expanding this idea to *Q. fragosa* vs. *Q. quadrula* species, the amount of genetic divergence is approximately 209 times less! This finding supports the distinction of *Q. fragosa* from *Q. quadrula*.

In summary our study supports the recognition of *Quadrula fragosa* as a distinct species from *Q. quadrula*, and that the *Q. fragosa* morphotypes sample from the four southern populations are genetically *Q. fragosa.* These conclusions are vital to the USFWS to develop a scientifically-based conservation management plan. Results from this study are also important for considerations for reclassification to threatened status and, ultimately, the delisting of *Q. fragosa.* For example, the first criteria for the reclassification to threatened status in the USFWS Recovery Plan (Vaughan, 1997) states that there must be three discrete populations in at least two tributaries of the Mississippi River drainage. The other criteria for reclassification include three viable populations (using recruitment, population size, age and genetic structure), persistence (looking at longevity and population surveys), and long-term habitat protection (based on physical, chemical, and biological habitat, and harvest and toxic spill protection) (Vaughan, 1997). This study concluded that there are at least four populations of *Q. fragosa* in three tributaries to the Mississippi River, but further work needs

to be performed to assess the viability and persistence of the populations described in the other reclassification criteria.

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Figure Legends

Figure 1. Images of *Quadrula* specimens: a) *Quadrula quadrula;* b) *Q. fragosa,* St. Croix River; c) *Q. fragosa,* Saline River, Arkansas; d) *Q. fragosa,* Ouachita River, Arkansas; e) *Q. fragosa,* Little River, Oklahoma; and f) *Q. fragosa,* Bourbeuse River, Missouri.

Figure 2. Map of specimen localities and adjacent river systems. Location A) denotes the population found in the St. Croix River between Minnesota and Wisconsin, B) shows the Bourbeuse River population in Missouri, C) the Saline River population in Arkansas, D) the Ouachita River population in Arkansas, and E) the Little River population in Oklahoma.

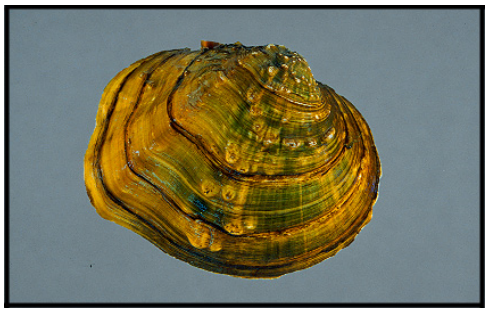
Figure 3. Phylogram of one of the 216 most parsimonious trees. Branch length represents amount of genetic difference.

Figure 4. Strict consensus of 216 trees recovered in maximum-parsimony analysis (tree length = 658, CI = 0.488, RI = 0.826). Numbers above the branches represent bootstrap support (100 addition replicates). Individuals of *Q. fragosa* are indicated with a dotted outline and by specific epithet. Other *Quadrula* species are indicated with the specific epithet. Non-*Quadrula* species are indicated with full species names. Species groups within *Quadrula* are labeled at appropriate nodes. Numbers included with each taxon are museum accession numbers (see Appendix A).

Figure 5. Majority rule tree using a Bayesian analysis. Numbers above branches represent posterior probabilities, with only posterior probabilities greater than 50% shown. The *Quadrula* genus is indicated with a black box, and the *quadrula, pustulosa,* and *metanevra* species groups with the spotted boxes. *Quadrula fragosa* is highlighted with a dotted outline.

Figure 1.

a)



b)

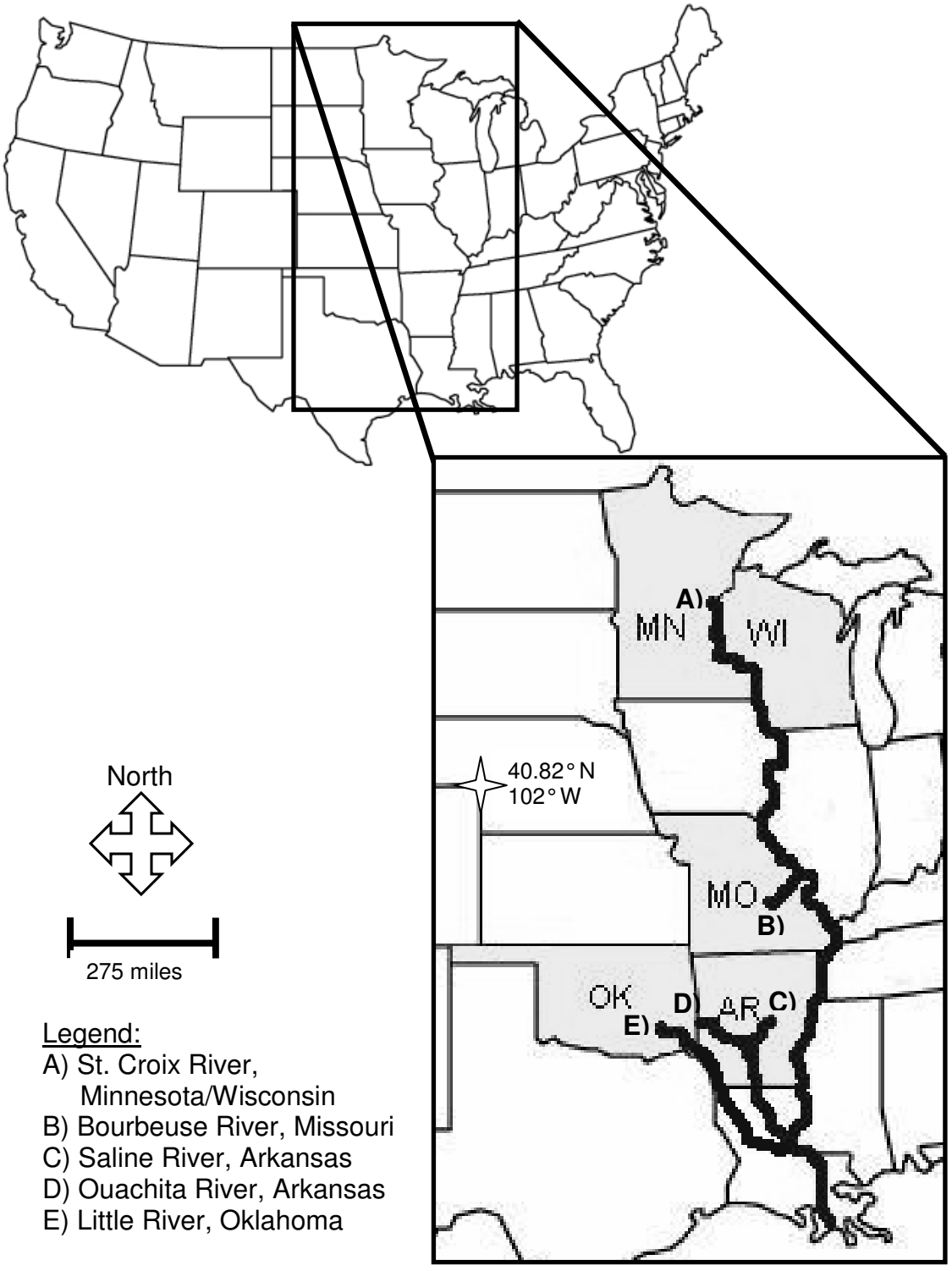
c)

d)

e)

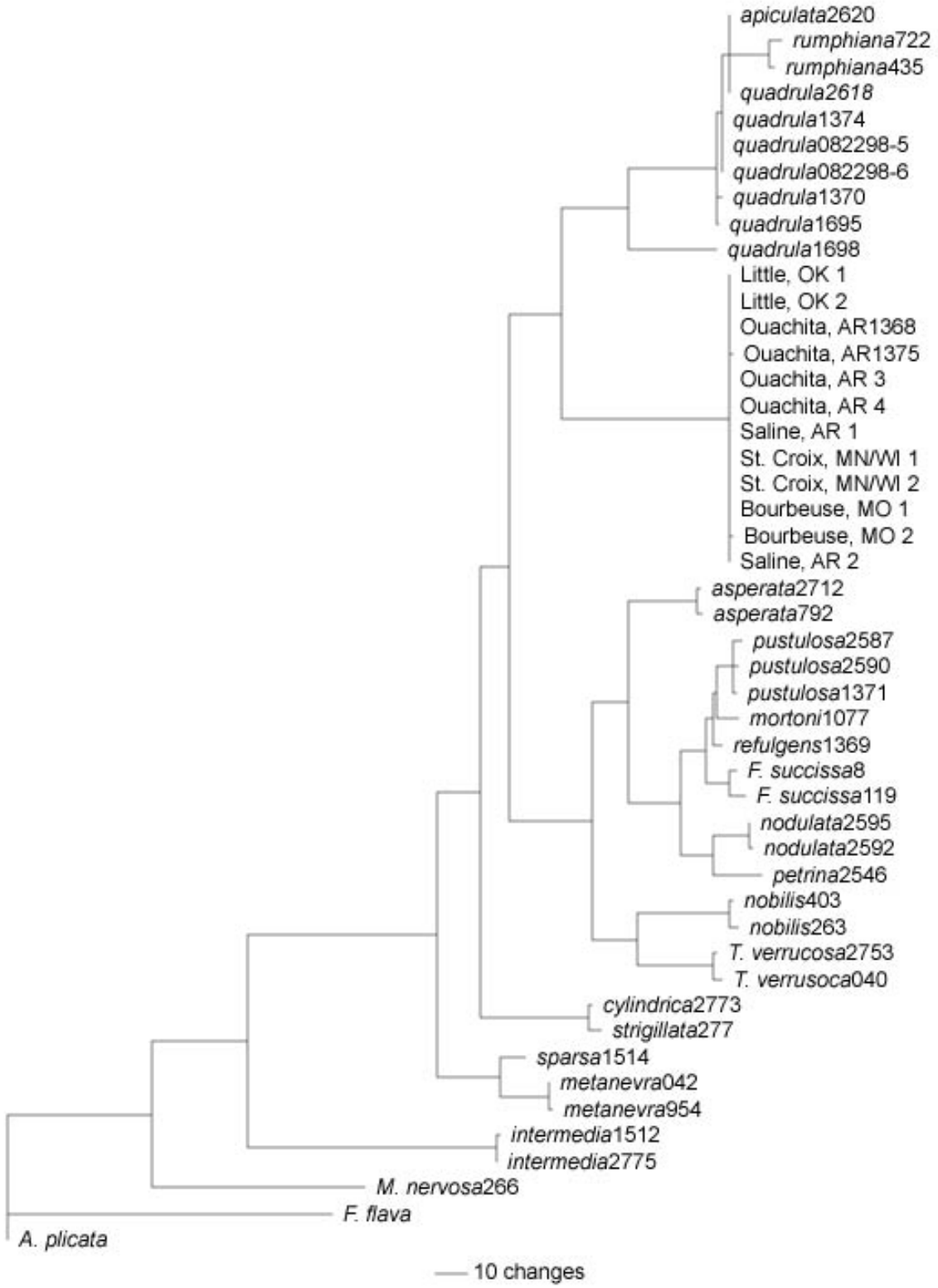
f)

Figure 2.



Legend:
A) St. Croix River, Minnesota/Wisconsin
B) Bourbeuse River, Missouri
C) Saline River, Arkansas
D) Ouachita River, Arkansas
E) Little River, Oklahoma

Figure 3.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rumphiana435
quadrula2618
quadrula1374
quadrula082298-5
quadrula082298-6
quadrula1370
quadrula1695
quadrula1698
Little, OK 1
Little, OK 2
Ouachita, AR1368
Ouachita, AR1375
Ouachita, AR 3
Ouachita, AR 4
Saline, AR 1
St. Croix, MN/WI 1
St. Croix, MN/WI 2
Bourbeuse, MO 1
Bourbeuse, MO 2
Saline, AR 2
asperata2712
asperata792
pustulosa2587
pustulosa2590
pustulosa1371
mortoni1077
refulgens1369
F. succissa8
F. succissa119
nodulata2595
nodulata2592
petrina2546
nobilis403
nobilis263
T. verrucosa2753
T. verrusoca040
cylindrica2773
strigillata277
sparsa1514
metanevra042
metanevra954
intermedia1512
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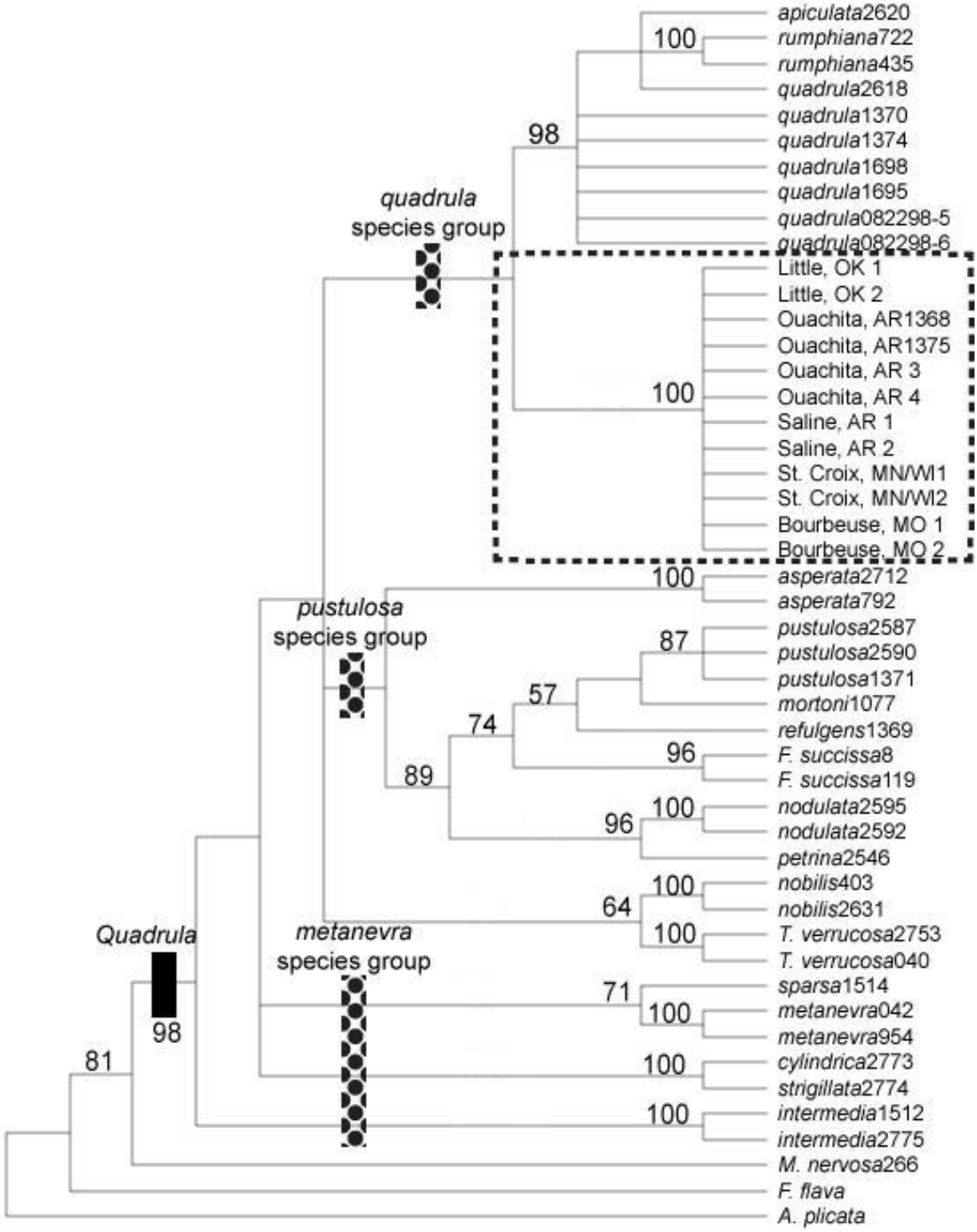
M. nervosa266
F. flava
A. plicata
10 changes

Figure 4.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quadrula082298-6
Little, OK 1
Little, OK 2
Ouachita, AR1368
Ouachita, AR1375
Ouachita, AR 3
Ouachita, AR 4
Saline, AR 1
Saline, AR 2
St. Croix, MN/WI1
St. Croix, MN/WI2
Bourbeuse, MO 1
Bourbeuse, MO 2
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asperata792
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pustulosa2590
pustulosa1371
mortoni1077
refulgens1369
F. succissa8
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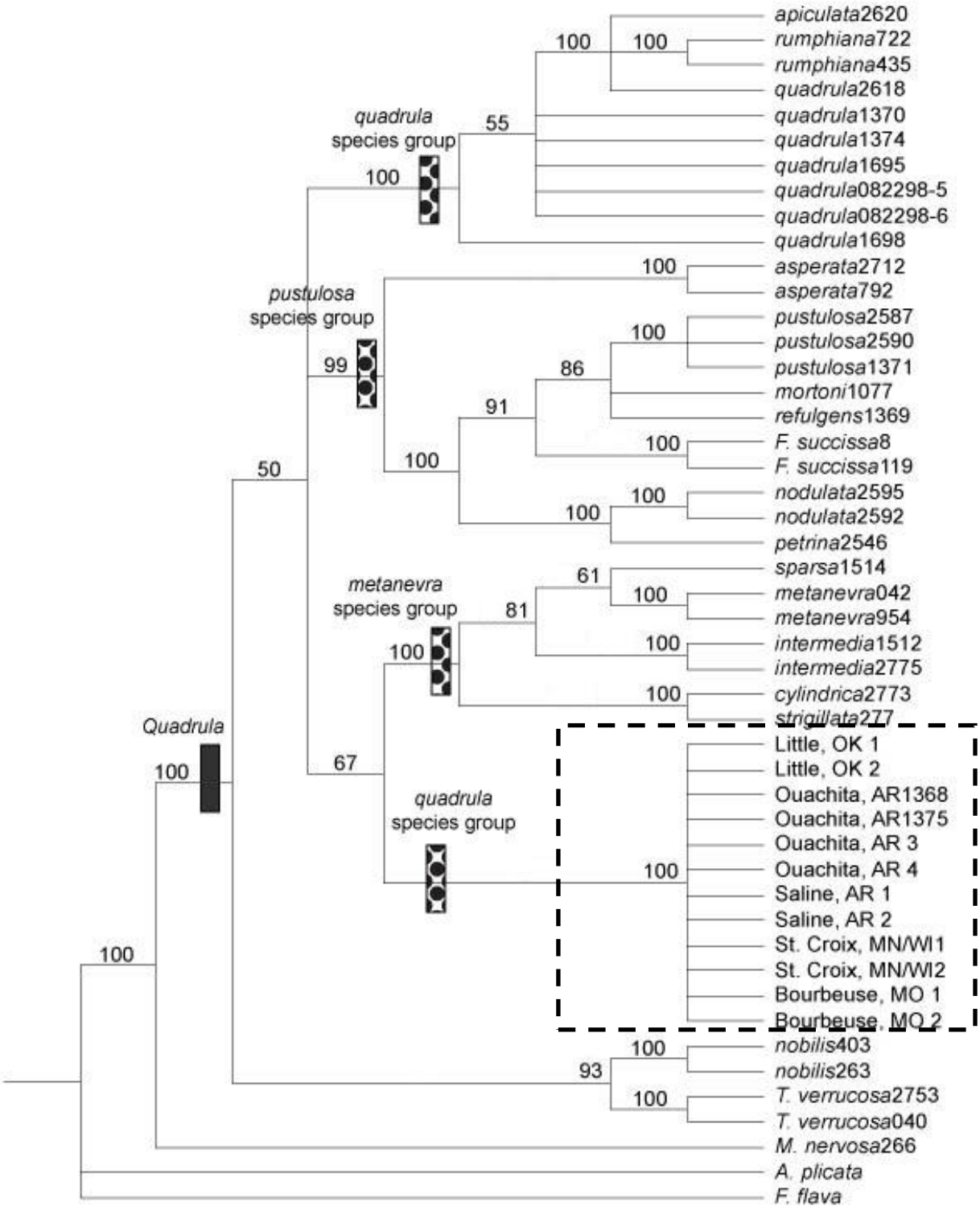
nodulata2595
nodulata2592
petrina2546
nobilis403
nobilis2631
T. verrucosa2753
T. verrucosa040
sparsa1514
metanevra042
metanevra954
cylindrica2773
strigillata2774
intermedia1512
intermedia2775
M. nervosa266
F. flava
A. plicata
quadrula species group
pustulosa species group
metanevra species group
Quadrula
100
98
100
87
57
74
96
89
100
96
100
64
100
71
100
100
98
81

Figure 5.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rumphiana722
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quadrula1698
asperata2712
asperata792
pustulosa2587
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pustulosa1371
mortoni1077
refulgens1369
F. succissa8
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nodulata2595
nodulata2592
petrina2546
sparsa1514
metanevra042
metanevra954
intermedia1512
intermedia2775
cylindrica2773
strigillata277
Little, OK 1
Little, OK 2
Ouachita, AR1368
Ouachita, AR1375
Ouachita, AR 3
Ouachita, AR 4
Saline, AR 1
Saline, AR 2
St. Croix, MN/WI1
St. Croix, MN/WI2
Bourbeuse, MO 1
Bourbeuse, MO 2
nobilis403
nobilis263
T. verrucosa2753
T. verrucosa040
M. nervosa266
A. plicata
F. flava
quadrula species group
pustulosa species group
metanevra species group
quadrula species group
Quadrula
100
100
55
100
100
100
99
86
91
100
100
50
100
100
61
100
81
100
100
100
67
100
100
93
100

Table 1. Specimens used with species name, museum number, GenBank number, locality, and collector. Voucher specimens are deposited at the University of Alabama Unionid Collection (UAUC) or the Illinois Natural History Survey (INHS). Localities are described by county (Co.) and/or river (R.) and state.

Species	Museum #	GenBank #	Locality	Collector
Q. quadrula	UAUC 1695	AY158774	Ohio R., Vanderburgh Co., Indiana	M. Smith
Q. quadrula	UAUC 1698	AY158773	Spring R., Cherokee Co., Kansas	M. Smith
Q. quadrula	ASUMZ 2618	None	Norwood Cr., McCurtain Co, Oklahoma	Crump, A.J. Harris, D. Arbour
Q. quadrula	1370 quad	None	AR Hwy 1, Bayou Macon, Desha Co., Arkansas	J. L. Harris, J. T. Fleming
Q. quadrula	MCB082298-05	None	Sac R., Cedar Co., Missouri	M.C. Barnhart
Q. quadrula	MCB082298-06	None	Sac R., Cedar Co.,Missouri	M.C. Barnhart
Q. fragosa	A19OK	None	Little River, Oklahoma	C. Vaughn
Q. fragosa	A21OK	None	Little River, Oklahoma	C. Vaughn
Q. fragosa	JS1368	None	Ouachita R., Ouachita Co., Arkansas	J.L. Harris
Q. fragosa	JS1375Q	None	Ouachita R., Ouachita Co., Arkansas	J.L. Harris
Q. fragosa	Af117	None	Tulip Creek, Ouachita River, Arkansas	J. L. Harris
Q. fragosa	Af120	None	Tulip Creek, Ouachita River, Arkansas	J. L. Harris
Q. fragosa	Af131	None	Saline River, Ashley-Bradly Co, Arkansas	C. Davidson
Q. fragosa	Af135	None	Saline River, Ashley-Bradly Co, Arkansas	C. Davidson
Q. fragosa	Af30	None	St. Croix River, Minnesota/Wisconsin	N. Rowse, P. Delphey
Q. fragosa	Af34	None	St. Croix River, Minnesota/Wisconsin	N. Rowse, P. Delphey
Q. fragosa	J318MO	None	Bourbeuse River, Franklin Co, Missouri	A. Roberts, S. McMurry
Q. fragosa	Af215	None	Bourbeuse River, Franklin Co, Missouri	J.M. Serb, K.J. Roe

Table 2. Calculations of genetic distances using the GTR+I+G (General Time Reversal + Invariant site + Gamma distribution of rate) model determined by MrModeltest v. 2.2.

Calculation of averages and ranges of genetic distance				
	Average	Percentage	Minimum	Maximum
within *Q. quadrula*	0.0032	0.315%	0.0000	0.0070
within *Q. fragosa*	0.0006	0.060%	0.0000	0.0037
Q. fragosa vs. *Q. quadrula*	0.1254	12.542%	0.1224	0.1312
Q. fragosa vs. *Q. quadrula* group	0.1319	13.186%	0.1224	0.1619
Q. fragosa vs. *Q. pustulosa* group	0.1770	17.704%	0.1464	0.2154
Q. fragosa vs. *Q. metanevra* group	0.1827	18.270%	0.1625	0.2104

APPENDIX A - MATERIALS EXAMINED

Voucher specimens are deposited at the University of Alabama Unionid Collection (UAUC), Arkansas State University Museum (ASUM), the Illinois Natural History Survey (INHS), the Ohio State University Museum of Biological Diversity (OSUM), or M. Christopher Barnhart (MCB). Museum catalog numbers, GenBank accession numbers (in parentheses), localities, and collectors are as follows: ***Quadrula apiculata*:** UAUC 2620 (AY158805) Neches River, Tyler County, Texas, R. G. Howells. ***Q. asperata*:** UAUC 792 (AY158757) Alabama River, Wilcox County, Alabama, J. T. Garner & P. D. Hartfield. UAUC 2712 (AY158768) Sucarnoochie Creek, Kemper County, Mississippi, S. J. Fraley & J. T. Baxter. ***Q. c. cylindrica*:** UAUC 2773 (AY158785) Duck River, Marshall County, Tennessee, S. J. Ahlstedt. ***Q. c. strigillata*:** UAUC 2774 (AY158800) Clinch River, Hancock County, Tennessee, S. J. Ahlstedt. ***Q. intermedia*:** UAUC 1512 (AY158760) Powell River, Lee County, Virginia, S. J. Ahlstedt & S. J. Fraley. UAUC 2772 (AY158782) and UAUC 2775 (AY158783) Duck River, Marshall County, Tennessee, S. J. Ahlstedt. ***Q. metanevra*:** UAUC 42 (AY158771) Elk River, Limestone County, Alabama, K. J. Roe. UAUC 954 (AY158803) Tennessee River, Hardin County, Tennessee, J. T. Garner & D. Hubbs. ***Q. mortoni*:** UAUC 1077 (AY158764) Big Cypress Bayou, Marion County, Texas, R. G. Howells. ***Q. nobilis*:** UAUC 403 (AY158786) Pascagoula River, Jackson County, Mississippi, D. N. Shelton. UAUC 2631 (AY158804) Neches River, Tyler County, Texas, R. G. Howells. ***Q. nodulata*:** UAUC 2592 (AY158756) Mississippi River, Marion County, Missouri, B. Sietman. UAUC 2595 (AY158755) Neches River, Tyler County, Texas, R. G. Howells. ***Q. petrina*:** UAUC 2546 (AY158798) Concho River, Concho County, Texas, R. G. Howells. ***Q. pustulosa*:** UAUC 2587 (AY158752) and Ouachita River, Ouachita County, Arkansas, J. L. Harris.

UAUC 2590 (AY158754) Mississippi River, Marion County, Missouri, B. Sietman. UAUC 1371 pust = Arkansas Hwy 1, Bayou Macon, Desha County, Arkansas, J. L. Harris and J. T. Fleming. ***Q. quadrula*:** UAUC 1695 (AY158774) Ohio River, Vanderburgh County, Indiana, M. Smith. UAUC 1698 (AY158773) Spring River, Cherokee County, Kansas, M. Smith. ASUMZ 2618 = (J435) Norwood Creek, McCurtain County, Oklahoma, J. L. Harris, B. G. Crump, A. J. Harris, D. Arbour. UAUC 3095 (J1370) quad Arkansas Hwy 1, Bayou Macon, Desha County, Arkansas, J. L. Harris and J. T. Fleming. **MCB082298-05** = (J320F) = *Q. quadrula* female Sac River, Missouri = quad4 (*Q. quadrula*) Sac River, Cedar County, Missouri, M.C. Barnhart. **MCB082298-06** = (J321) = *Q. quadrula* male Sac River, Missouri = quad5 (*Q. quadrula*) Sac River, Cedar County, Missouri, M.C. Barnhart. ***Q. refulgens*:** UAUC 405 (AY158788) Pascagoula River, Jackson County, Mississippi, D. N. Shelton. JS1369 **=** Arkansas Hwy 1, Bayou Macon, Desha County, Arkansas, J. L. Harris and J. T. Fleming. ***Q. rumphiana*:** UAUC 435 (AY158776) Oostanaula River, Gordon County, Georgia, J. D. Williams. UAUC 722 (AY158775) Sipsey River, Pickens County, Alabama, H. McCullogh & C. Lydeard. ***Q. sparsa*:** UAUC 1514 (AY158761) Powell River, Lee County, Virginia, S. J. Ahlstedt & S. J. Fraley. ***Q. fragosa:*** A19OK (OK1), Little River, Oklahoma, C. Vaughn; A21OK (OK2), Little River, Oklahoma, C. Vaughn; JS1368, JS1375Q; Af117 (Ouachita, AR 3), Tulip Creek, Ouachita River, Arkansas, J. L. Harris; Af120 (Ouachita, AR 4), Tulip Creek, Ouachita River, Arkansas, J. L. Harris; Af131 (Saline, AR 1), Saline River, Ashley-Bradley County, Arkansas, C. Davidson; Af135 (Saline, AR 2), Saline River, Ashley-Bradley Co, Arkansas, C. Davidson; Af30 (St. Croix, MN/WI 1), St. Croix River, Minnesota/Wisconsin, N. Rowse and P. Delphey; Af34 (St. Croix, MN/WI 2), St. Croix River, Minnesota/Wisconsin, N. Rowse and P. Delphey; J318MO (Bourbeuse, MO

1), Bourbeuse River, Franklin County, Missouri, A. Roberts, S. McMurry; Af215 (Bourbeuse, MO 2), Bourbeuse River, Franklin County, Missouri, J.M. Serb and K.J. Roe.

Outgroup taxa: ***Amblema plicata*****:** UAUC 147 (AY158796) Ohio River, Kentucky, P. Morrison. ***Fusconaia flava*****:** UAUC 146 (AY158781) Ohio River, Kentucky, P. Morrison. ***F. succissa*****:** 8=UAUC 1456 (AY158792) Conecuh River, Pike County, Alabama, J. D. Williams. 119=UAUC 525 (AY158809) Pea River, Geneva County, Alabama, J. D. Williams. ***Megalonaias nervosa*****:** UAUC 266 (AY158794) Coosa River, Cherokee County, Alabama, K. J. Roe. ***Tritogonia verrucosa*****:** UAUC 40 (AY158791) Elk River, Limestone County, Alabama, K. J. Roe. UAUC 2753 (AY158807) Cumberland River, Scott County, Tennessee, S. J. Ahlstedt.

APPENDIX B - Pairwise genetic distances between taxa using the GTR+I+G (General Time Reversal + Invariant site + Gamma distribution of rate) model.

	apiculata 2620	*asperata* 2712	*asperata* 792	*pustulosa* 2587	*pustulosa* 2590	*pustulosa* 1371	*nodulata* 2595	*nodulata* 2592
apiculata 2620	-							
asperata 2712	0.13	-						
asperata 792	0.13	0.01	-					
pustulosa 2587	0.15	0.09	0.10	-				
pustulosa 2590	0.15	0.10	0.10	0.01	-			
pustulosa 1371	0.14	0.09	0.10	0.01	0.01	-		
nodulata 2595	0.17	0.11	0.11	0.07	0.08	0.07	-	
nodulata 2592	0.18	0.11	0.12	0.08	0.08	0.08	0.00	-
mortoni 1077	0.16	0.11	0.11	0.03	0.03	0.02	0.07	0.08
refulgens 1369	0.15	0.09	0.10	0.02	0.02	0.01	0.07	0.07
quadrula 1370	0.01	0.12	0.12	0.14	0.14	0.14	0.17	0.17
quadrula 1374	0.00	0.12	0.12	0.14	0.14	0.14	0.17	0.17
quadrula 1698	0.01	0.12	0.12	0.14	0.13	0.14	0.16	0.16
quadrula 1695	0.01	0.12	0.12	0.14	0.14	0.14	0.16	0.17
rumphiana 722	0.03	0.16	0.16	0.16	0.16	0.16	0.19	0.20
rumphiana 435	0.03	0.16	0.16	0.16	0.16	0.16	0.19	0.20
nobilis 403	0.14	0.15	0.15	0.16	0.16	0.16	0.17	0.18
nobilis 2631	0.14	0.16	0.16	0.17	0.17	0.17	0.19	0.19
petrina 2546	0.19	0.14	0.14	0.08	0.08	0.08	0.05	0.05
sparsa 1514	0.16	0.13	0.14	0.14	0.14	0.14	0.15	0.16
intermedia 1512	0.22	0.21	0.21	0.19	0.20	0.19	0.21	0.22
intermedia 2775	0.22	0.20	0.21	0.19	0.20	0.20	0.22	0.22
cylindrica 2773	0.15	0.15	0.15	0.17	0.18	0.18	0.20	0.20
strigillata 2774	0.16	0.15	0.16	0.17	0.17	0.17	0.19	0.19
metanevra 042	0.17	0.14	0.15	0.16	0.16	0.16	0.18	0.18
metanevra 954	0.18	0.15	0.15	0.16	0.17	0.16	0.18	0.18
F.succissa 8	0.15	0.09	0.10	0.04	0.04	0.03	0.07	0.07
T.verrucosa 2753	0.15	0.15	0.15	0.13	0.14	0.13	0.16	0.17
M.nervosa 266	0.34	0.34	0.34	0.28	0.28	0.29	0.28	0.28
A.plicata	0.39	0.36	0.37	0.39	0.39	0.40	0.39	0.40
F.flava	0.33	0.37	0.36	0.41	0.40	0.42	0.42	0.43
F.succissa 119	0.16	0.11	0.11	0.04	0.04	0.04	0.08	0.08
T.verrucosa 040	0.15	0.15	0.16	0.14	0.14	0.14	0.16	0.17
quadrula 082298-5	0.00	0.12	0.12	0.14	0.14	0.14	0.17	0.17
quadrula 082298-6	0.00	0.12	0.12	0.14	0.14	0.14	0.17	0.17
quadrula 2618	0.00	0.13	0.13	0.15	0.15	0.14	0.17	0.18
Little, OK 1	0.13	0.16	0.17	0.16	0.17	0.16	0.20	0.21
Little, Ok 2	0.13	0.16	0.17	0.16	0.17	0.16	0.20	0.21
Ouachita, AR1368	0.13	0.16	0.17	0.16	0.17	0.16	0.20	0.21
Ouachita, AR1375	0.13	0.17	0.18	0.17	0.17	0.16	0.21	0.21
Ouachita, AR 3	0.13	0.16	0.17	0.16	0.17	0.16	0.20	0.21
Ouachita, AR 4	0.13	0.16	0.17	0.16	0.17	0.16	0.20	0.21
Saline, AR 1	0.12	0.16	0.17	0.16	0.17	0.16	0.20	0.20
Saline, AR 2	0.13	0.16	0.17	0.16	0.17	0.16	0.20	0.21
St. Croix, MN/WI1	0.13	0.16	0.17	0.16	0.17	0.16	0.20	0.21
St. Croix, MN/WI2	0.13	0.16	0.17	0.16	0.17	0.16	0.20	0.21
Bourbeuse, MO 1	0.13	0.16	0.17	0.16	0.17	0.16	0.20	0.21
Bourbeuse, MO 2	0.12	0.15	0.16	0.15	0.16	0.15	0.19	0.19

APPENDIX B (continued)

	mortoni 1077	*refulgens* 1369	*quadrula* 1370	*quadrula* 1374	*quadrula* 1698	*quadrula* 1695	*rumphiana* 722	*rumphiana* 435
mortoni 1077	-							
refulgens 1369	0.02	-						
quadrula 1370	0.15	0.14	-					
quadrula 1374	0.15	0.14	0.00	-				
quadrula 1698	0.14	0.14	0.00	0.00	-			
quadrula 1695	0.15	0.14	0.01	0.00	0.00	-		
rumphiana 722	0.17	0.17	0.04	0.03	0.03	0.03	-	
rumphiana 435	0.17	0.16	0.03	0.03	0.03	0.03	0.01	-
nobilis 403	0.16	0.15	0.14	0.14	0.13	0.13	0.17	0.17
nobilis 2631	0.18	0.16	0.14	0.14	0.14	0.14	0.18	0.17
petrina 2546	0.08	0.07	0.18	0.18	0.18	0.18	0.21	0.20
sparsa 1514	0.15	0.14	0.15	0.15	0.14	0.15	0.17	0.17
intermedia 1512	0.20	0.20	0.22	0.21	0.21	0.21	0.23	0.23
intermedia 2775	0.21	0.21	0.22	0.21	0.21	0.21	0.22	0.23
cylindrica 2773	0.20	0.18	0.15	0.14	0.14	0.14	0.16	0.16
strigillata 2774	0.19	0.17	0.16	0.15	0.15	0.15	0.16	0.16
metanevra 042	0.17	0.17	0.17	0.17	0.16	0.17	0.17	0.18
metanevra 954	0.18	0.17	0.17	0.17	0.17	0.17	0.18	0.18
F.succissa 8	0.03	0.03	0.14	0.14	0.13	0.14	0.17	0.17
T.verrucosa 2753	0.14	0.14	0.15	0.15	0.14	0.15	0.15	0.16
M.nervosa 266	0.31	0.29	0.34	0.33	0.33	0.33	0.34	0.33
A.plicata	0.43	0.40	0.40	0.39	0.39	0.38	0.40	0.39
F.flava	0.44	0.42	0.34	0.33	0.33	0.33	0.35	0.35
F.succissa 119	0.04	0.03	0.15	0.15	0.15	0.15	0.18	0.18
T.verrucosa 040	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.15
quadrula 082298-5	0.15	0.14	0.00	0.00	0.00	0.00	0.03	0.03
quadrula 082298-6	0.15	0.14	0.00	0.00	0.00	0.00	0.03	0.03
quadrula 2618	0.16	0.15	0.01	0.00	0.01	0.01	0.03	0.03
Little, OK 1	0.18	0.17	0.12	0.12	0.12	0.13	0.16	0.15
Little, Ok 2	0.18	0.17	0.12	0.12	0.12	0.13	0.16	0.15
Ouachita, AR1368	0.18	0.17	0.12	0.12	0.12	0.13	0.16	0.15
Ouachita, AR1375	0.18	0.17	0.13	0.13	0.13	0.13	0.16	0.15
Ouachita, AR 3	0.18	0.17	0.12	0.12	0.12	0.13	0.16	0.15
Ouachita, AR 4	0.18	0.17	0.12	0.12	0.12	0.13	0.16	0.15
Saline, AR 1	0.18	0.16	0.12	0.12	0.12	0.13	0.16	0.15
Saline, AR 2	0.18	0.17	0.12	0.12	0.12	0.13	0.16	0.15
St. Croix, MN/WI1	0.18	0.17	0.12	0.12	0.12	0.13	0.16	0.15
St. Croix, MN/WI2	0.18	0.17	0.12	0.12	0.12	0.13	0.16	0.15
Bourbeuse, MO 1	0.18	0.17	0.12	0.12	0.12	0.13	0.16	0.15
Bourbeuse, MO 2	0.16	0.15	0.12	0.12	0.12	0.13	0.15	0.15

APPENDIX B (continued)

	nobilis 403	*nobilis* 2631	*petrina* 2546	*sparsa* 1514	*intermedia* 1512	*intermedia* 2775	*cylindrica* 2773	*strigillata* 2774
nobilis 403	-							
nobilis 2631	0.01	-						
petrina 2546	0.20	0.21	-					
sparsa 1514	0.15	0.15	0.16	-				
intermedia 1512	0.20	0.21	0.22	0.09	-			
intermedia 2775	0.21	0.21	0.22	0.09	0.00	-		
cylindrica 2773	0.19	0.20	0.20	0.09	0.14	0.14	-	
strigillata 2774	0.19	0.20	0.19	0.10	0.14	0.14	0.01	-
metanevra 042	0.19	0.19	0.19	0.04	0.11	0.11	0.12	0.12
metanevra 954	0.20	0.19	0.19	0.05	0.11	0.11	0.11	0.12
F.succissa 8	0.16	0.17	0.08	0.15	0.22	0.22	0.18	0.18
T.verrucosa 2753	0.11	0.12	0.19	0.15	0.20	0.21	0.19	0.18
M.nervosa 266	0.35	0.34	0.29	0.30	0.33	0.32	0.32	0.31
A.plicata	0.38	0.38	0.38	0.41	0.36	0.37	0.40	0.39
F.flava	0.38	0.38	0.40	0.37	0.40	0.39	0.37	0.37
F.succissa 119	0.16	0.17	0.09	0.15	0.21	0.22	0.18	0.18
T.verrucosa 040	0.12	0.13	0.19	0.15	0.21	0.21	0.19	0.18
quadrula 082298-5	0.14	0.14	0.18	0.15	0.21	0.21	0.14	0.15
quadrula 082298-6	0.14	0.14	0.18	0.15	0.21	0.21	0.14	0.15
quadrula 2618	0.14	0.14	0.19	0.16	0.22	0.22	0.15	0.16
Little, OK 1	0.21	0.20	0.21	0.17	0.20	0.21	0.17	0.17
Little, Ok 2	0.21	0.20	0.21	0.17	0.20	0.21	0.17	0.17
Ouachita, AR1368	0.21	0.20	0.21	0.17	0.20	0.21	0.17	0.17
Ouachita, AR1375	0.22	0.21	0.22	0.17	0.21	0.21	0.18	0.18
Ouachita, AR 3	0.21	0.20	0.21	0.17	0.20	0.21	0.17	0.17
Ouachita, AR 4	0.21	0.20	0.21	0.17	0.20	0.21	0.17	0.17
Saline, AR 1	0.21	0.20	0.20	0.16	0.20	0.20	0.17	0.17
Saline, AR 2	0.21	0.20	0.21	0.17	0.20	0.21	0.17	0.17
St. Croix, MN/WI1	0.21	0.20	0.21	0.17	0.20	0.21	0.17	0.17
St. Croix, MN/WI2	0.21	0.20	0.21	0.17	0.20	0.21	0.17	0.17
Bourbeuse, MO 1	0.21	0.20	0.21	0.17	0.20	0.21	0.17	0.17
Bourbeuse, MO 2	0.21	0.21	0.19	0.16	0.20	0.20	0.17	0.17

APPENDIX B (continued)

	metanevra 042	*metanevra* 954	*F.succissa* 8	*T.verrucosa* 2753	*M.nervosa* 266	*A.plicata*	*F.flava*	*F.succissa* 119
metanevra 042	-							
metanevra 954	0.00	-						
F.succissa 8	0.19	0.19	-					
T.verrucosa 2753	0.17	0.17	0.14	-				
M.nervosa 266	0.32	0.33	0.32	0.31	-			
A.plicata	0.40	0.40	0.42	0.44	0.33	-		
F.flava	0.37	0.38	0.43	0.43	0.33	0.27	-	
F.succissa 119	0.18	0.18	0.01	0.14	0.30	0.42	0.42	-
T.verrucosa 040	0.17	0.17	0.15	0.01	0.31	0.45	0.42	0.15
quadrula 082298-5	0.17	0.17	0.14	0.15	0.33	0.39	0.33	0.15
quadrula 082298-6	0.17	0.17	0.14	0.15	0.33	0.39	0.33	0.15
quadrula 2618	0.17	0.18	0.15	0.15	0.34	0.39	0.33	0.16
Little, OK 1	0.18	0.18	0.17	0.21	0.37	0.39	0.42	0.18
Little, Ok 2	0.18	0.18	0.17	0.21	0.37	0.39	0.42	0.18
Ouachita, AR1368	0.18	0.18	0.17	0.21	0.37	0.39	0.42	0.18
Ouachita, AR1375	0.18	0.18	0.17	0.21	0.38	0.40	0.42	0.18
Ouachita, AR 3	0.18	0.18	0.17	0.21	0.37	0.39	0.42	0.18
Ouachita, AR 4	0.18	0.18	0.17	0.21	0.37	0.39	0.42	0.18
Saline, AR 1	0.17	0.18	0.17	0.20	0.37	0.39	0.41	0.17
Saline, AR 2	0.18	0.18	0.17	0.21	0.37	0.39	0.42	0.18
St. Croix, MN/WI1	0.18	0.18	0.17	0.21	0.37	0.39	0.42	0.18
St. Croix, MN/WI2	0.18	0.18	0.17	0.21	0.37	0.39	0.42	0.18
Bourbeuse, MO 1	0.18	0.18	0.17	0.21	0.37	0.39	0.42	0.18
Bourbeuse, MO 2	0.17	0.18	0.15	0.19	0.37	0.38	0.41	0.16

APPENDIX B (continued)

	T.verrucosa 040	*quadrula* 082298-5	*quadrula* 082298-6	*quadrula* 2618	Little, OK 1	Little, Ok 2	Ouachita, AR1368	Ouachita, AR1375
T.verrucosa 040	-							
quadrula 082298-5	0.14	-						
quadrula 082298-6	0.14	0.00	-					
quadrula 2618	0.15	0.00	0.00	-				
Little, OK 1	0.21	0.13	0.12	0.13	-			
Little, Ok 2	0.21	0.13	0.12	0.13	0.00	-		
Ouachita, AR1368	0.21	0.13	0.12	0.13	0.00	0.00	-	
Ouachita, AR1375	0.21	0.13	0.13	0.13	0.00	0.00	0.00	-
Ouachita, AR 3	0.21	0.13	0.12	0.13	0.00	0.00	0.00	0.00
Ouachita, AR 4	0.21	0.13	0.12	0.13	0.00	0.00	0.00	0.00
Saline, AR 1	0.21	0.12	0.12	0.12	0.00	0.00	0.00	0.00
Saline, AR 2	0.21	0.13	0.12	0.13	0.00	0.00	0.00	0.00
St. Croix, MN/WI1	0.21	0.13	0.12	0.13	0.00	0.00	0.00	0.00
St. Croix, MN/WI2	0.21	0.13	0.12	0.13	0.00	0.00	0.00	0.00
Bourbeuse, MO 1	0.21	0.13	0.12	0.13	0.00	0.00	0.00	0.00
Bourbeuse, MO 2	0.19	0.12	0.12	0.12	0.00	0.00	0.00	0.00

APPENDIX B (continued)

	Ouachita, AR 3	Ouachita, AR 4	Saline, AR 1	Saline, AR 2	St. Croix, MN/WI1	St. Croix, MN/WI2	Bourbeuse, MO 1	Bourbeuse, MO 2
Ouachita, AR 3	-							
Ouachita, AR 4	0.00	-						
Saline, AR 1	0.00	0.00	-					
Saline, AR 2	0.00	0.00	0.00	-				
St. Croix, MN/WI1	0.00	0.00	0.00	0.00	-			
St. Croix, MN/WI2	0.00	0.00	0.00	0.00	0.00	-		
Bourbeuse, MO 1	0.00	0.00	0.00	0.00	0.00	0.00	-	
Bourbeuse, MO 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-

CHAPTER 3 - DEVELOPMENT AND CHARACTERIZATION OF TWELVE MICROSATELLITE MARKERS FOR THE ENDANGERED WINGED-MAPLELEAF, *QUADRULA FRAGOSA* (BIVALVIA: UNIONIDAE)

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Abstract

Primers for twelve microsatellite loci were developed for the endangered winged mapleleaf, *Quadrula fragosa,* from the St. Croix River between Minnesota and Wisconsin. The markers were tested and characterized using individuals from five separate populations: St. Croix River, MN/WI; Bourbeuse River, MO; Saline river and Ouachita rivers, AR; and Little River, OK. Allelic diversity was used to analyze the variation within each population and among the five populations. With the range expansion, the results from this study will be used in an effort to conserve the species via potential captive breeding programs and/or translocation efforts. Our study resulted in 18 developed primer sets; 12 of which consistently amplified and 9 of which were polymorphic across all individuals. Allelic diversity ranged from 1-28, with repeat sizes from 160-473 base pairs. Observed (H_o) heterozygosity ranged from 0.040-0.692, and expected (H_e) heterozygosity ranged from 0.036-0.838.

One freshwater mussel species in jeopardy of extinction is the winged-mapleleaf, *Quadrula fragosa*. It was historically found in ten to twelve states across the US, in the Mississippi, Tennessee, Ohio, and Cumberland River drainages (Posey *et al*., 1996). Due to habitat degradation, the species was believed to be reduced to a single population found in the St. Croix River between Minnesota and Wisconsin, which resulted in an endangered listing by the US Fish and Wildlife Service in 1991 (USFWS, 1991; Hornbach *et al.*, 1996). This study designed 12 amplifiable primers to be used in a population genetics study to aid in conservation management decisions.

Samples of mantle tissue were biopsied from individuals from the St. Croix, Bourbeuse, Saline, Ouachita, and Little rivers (Table 3). Total genomic DNA was extracted using the spin-column protocol for animal tissues of the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, California). DNA integrity was determined using a 1% agarose gel, and concentration was determined with UV fluorescence using NanoDrop, model ND-1000.

The procedure of Kandpal *et al*. (1994) was used to create an enriched genomic library for $(CA)_n$ repeats. Briefly, approximately 5 µg of genomic DNA was digested using the *Sau*3AI restriction enzyme. The digested DNA was size fractionated using Chroma Spin (Clonetech Laboratories) to create a pool of fragments ranging from 0.4-1.5 kb in length. *Sau*3AI oligonucleotide linkers were ligated to the size selected DNA and these linker-ligated fragments were amplified via PCR for 25 cycles using primers complementary to the linker sequence. $(CA)_{15}$ biotinylated probes were used to capture repeat containing fragments using the VECTREX Avidin D matrix (Vector Laboratories, Burlingame, CA). Following elution of the captured repeat containing fragments, the library was enriched by another round of PCR using the same primers as before.

"In-house" microsatellite development was performed using the enriched library. Transformed *E. coli* cells were plated on LB+Amp plates containing X-gal (40 mg/mL) and incubated for 12 hours. Clones were isolated for –CA repeats using a colony lift hybridization protocol to extract the DNA, denature it, and affix to a nylon membrane. Then, the membrane was hybridized with a biotin label, and detected using the Phototope™-Star Detection Kit for Nucleic Acids by New England Biolabs, Inc (New England Biolabs, Massachusetts). Positive colonies containing repeats were colonies that showed on the X-ray film and were white. These were selected and placed in 750 µl of TB+Amp media, and incubated for approximately 15 hours. The Qiagen QIAprep® Spin Miniprep Kit (Qiagen, California) was used to isolate the plasmid DNA. To determine whether the plasmid contained the desired repeat insert, each sample was restriction digested with EcoRI, and visualized on a 1% agarose gel. Samples with a band size greater than 400 base pairs were selected for sequencing. Applied Biosystem's PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 was used for sequencing reactions. The sequenced samples were prepared using an ethanol/EDTA precipitation protocol, and submitted to the DNA Facility of the Iowa State University Office of Biotechnology. Primers were designed for individuals displaying repeated motifs. Eighteen primer sets were designed, with repeats ranging from 3-52, using online software from Integrated DNA Technologies. Primers were hydrated with water for a concentration of 100 µM, and then diluted to 10 µM for a working stock.

Genetic Identification Services of Chatsworth, California (GIS) was recruited for additional markers. Approximately 30.8 ng of genomic DNA, suspended in 100 µl TE

buffer, was submitted to GIS. GIS provided primer sequences for selected individuals showing di and tetra repeats.

GIS supplied sequences for 54 additional primers, for a total of 74. The annealing temperature for each primer set was optimized for genotyping via PCR. Reagents included 1µl of each primer at 10µM, 2.5µl each of 10X Buffer and 50 mM $MgCl_2$, 16.25 µl water, 0.5 µl 10 mM dNTP's, 0.25 µl *Taq*, and 1 µl DNA. PCR conditions were 35 cycles of 96°C for 30 sec, 10°C gradient for 30 sec, 72°C for 30 sec, followed by 72°C for 3 min, and a 10°C hold. Once the optimal temperature was determined, each primer set underwent another PCR (same as above), was sequenced using the same method mentioned previously, and compared to the original primer design for matching repeat and flanking sequences. If the sequence was very similar to identical, then it was used to screen for repeat length variation in each individual from each of the five populations. Variation of band size and number was visualized using a 3% NuSieve agarose gel. After checking for amplification, confirming the correct sequence, and checking for initial size variation, this study was left with 12 primers to use in genotyping each individual. Individuals of each population were genotyped with each of the 12 primers. The PCR mix included 0.07 µl forward primer with attached M13 sequence, 1µl each of the regular reverse and M13-dye labeled primers, 2.5 µl 10X Buffer (with $MgCl_2$ included), 18.68 µl water, 0.5µl 10 mM dNTP's, 0.25µl high-fidelity *Taq* polymerase, and 1µl of 25-30 ng/µl of DNA. PCR conditions were 95° for 5 min, 7 cycles of 96°C for 30 sec, 67-55°C for 90 sec with the temperature decreasing by 2°C each cycle, and 72°C for 60 sec. The reaction continued with 27 cycles of 94°C for 30 sec, 55°C for 90 sec, 72°C for 60 sec., followed by a final extension at 60°C for 30 min and a 10°C hold. A random subset was visualized on a 3% NuSieve agarose gel to ensure the reactions worked.

Then, 1.5 µl of each sample was submitted to the Iowa State University DNA Facility where they were visualized using an ABI 3100 Analyzer.

Peaks were analyzed for allelic size and genotype using Genemapper v.4 software by Applied Biosystems. The program GenAlEx (Peakall and Smouse, 2006) was used to calculate the observed and expected heterozygosities. A chi-square test was performed to calculate a p-value to determine deviations from Hardy-Weinberg equilibrium (HWE).

Primer development resulted in 12 amplifiable primer sets (Table 1). Out of the twelve amplifiable primers, nine were polymorphic across all individuals (A103, A112, A130, C4, C109, C114, D102, N9, and R9), and three were monomorphic (N8, H8, and Q1A). Forty-three individuals (Table 3) were genotyped using the method developed by Schuelke (2000). Allelic diversity ranged from 1-28, with an average of 8.25 alleles per locus. Size range of the repeats across all loci was 160-473 base pairs. Observed (H_o) heterozygosity ranged from 0.040-0.692, with an average of 0.389 across all loci. Expected (H_e) heterozygosity ranged from 0.036-0.838, with an average of 0.554 across all loci. These values are summarized in Table 2. Values were not significant per locus across all populations.

These primers will be used in a population genetic analysis to assess the structure of *Q. fragosa.* The results will be forwarded to the United States Fish and Wildlife Service and United States Army Corps of Engineers to develop appropriate conservation plans. The newly developed primers may also be used for future trials to cross-amplify with other *Quadrula* species.

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Table 1. Characteristics of 18 microsatellite loci. Fluorescently labeled primers (6-FAM) are in bold, the annealing temperature (°C), and repeat motif. *Primers not used due to poor PCR amplification.

Name	Primer Sequence	Anneal Temp	Repeat Motif
QfA103	**F-5'-GCA CAC CTT ATT CAT TTG AGA-3'**	49	CA
	R-5'-AAT GTC TTC CCC ATG ACT AAA-3'		
QfA112	**F-5'-ACT TGC TCC AAA ACT TGT AGA G-3'**	56	CA
	R-5'-GGA ATG GTT CAG ACT ATG ACC-3'		
QfA130	**F-5'-TGA GAA ATC GTG ATG ACT CAG-3'**	58	TG
	R-5'-CCT ACC TAC CTT CAT GTG GTC-3'		
QfC4	**F-5'-TGT CCT TCT CTG TGA ATG TTT G-3'**	58	TACA
	R-5'-GCA CTC CAT AAA TGC AGG TAA T-3'		
QfC109	**F-5'-GAC AGG AAA TAA AGG GTG TC-3'**	55	TATG
	R-5'-GCA ATG TAA TAT GGT ATG CAC-3'		
QfC114	**F-5'-TCC ATG TTT TTC TCC TCC TCT A-3'**	58	TACA
	R-5'-CAC CCT TGC TTA TAG CGT AGT C-3'		
QfD102	**F-5'-TGG ACA ATT CAT CAA GTC AAG-3'**	53	ATCT
	R-5'-CTT TGT TTT CCA AAC CAT ACA G-3'		
N8	**F-5'-AGC TTG GGA TCA CCT ATG ACC C-3'**	63	CA
	R-5'-GCC CTT CAG ACA GTG TCC TCT CTG CT-3'		
N9	**F-5'-TCG TCT ACC ACC TCT GCA ACA CAT ACC G-3'**	68	TG
	R-5'-GGC AGA GAG GTC ACA ACC CCG GA-3'		
H8	**F-5'-ACC CTT GTG GGT GTG GTG TGG AGA ACG-3'**	68	CAA
	R-5'-GGA TCC AAT CGG AGA GCC TGA GGT-3'		
Q1A	**F-5'-ACA GTT CTA GTG TCC GAG GAG TCA CTG G-3'**	64	TTG
	R-5'-GGT GTA TTG TGT CAT CGG TGC TGC CA-3'		
R9	**F-5'-AGC TTG GGA TCG GAG TTG CAG CCA GC-3'**	66	CA
	R-5'-GGA CAC CCC AGT GTG TAA GAA CA-3'		
QfD2*	**F-5'-TGG ATG TTA TTG TGC TTA ACG A-3'**	50	TAGA
	R-5'-GCC ATT TAT CAA AGA ATG CAG-3'		
QfD103*	**F-5'-ACG TGT AAC CGA TTG GTA TAT C-3'**	55	TCTA
	R-5'-GTA TGA AGG GAC GAA AAT GTA C-3'		
QfD116*	**F-5'-CCA TGT AAA GGT TTG CAT TAA C-3'**	48	TAGA
	R-5'-TGG ACA CAC CAC ATA TAC AGA C-3'		
N11*	**F-5'-TGT GGC TGT GCT GGT GAC TCA TTT CC-3'**	68	CA
	R-5'-CCA TGC CAT CAG GTG CAG GA-3'		
O2*	**F-5'-AGC AGA CTT CAT CGA GAC AAA AAT GGT CGG-3'**	66	TG
	R-5'-CCA GTT CAT CAG TCG GTA TAT TCT TCC GCT-3'		
P5*	**F-5'-TCG CCA CGG TAC AAT CAG TTC TTG CAA CG-3'**	62	CAC
	R-5'-GCG TGT CTG ACG AGC AAT AGG T-3'		

Table 2. Results of microsatellite screen. Primers are listed with repeat motif, size range, number of alleles (A), observed and expected heterozygosities (H_O and H_E), p-value based on chi-square, and number sampled (n). *Primers not used due to poor PCR amplification.

Name	Repeat Motif	Size Range (bp)	A	H_o	H_e	p	F_{IS}	n
QfA103	CA	298-302	2	0.484	0.341	0.184	-0.421	43
QfA112	CA	160-180	10	0.327	0.654	0.157	0.500	42
QfA130	TG	287-323	14	0.489	0.720	0.423	0.321	43
QfC4	TACA	236-380	28	0.692	0.838	0.451	0.174	43
QfC109	TATG	177-285	10	0.463	0.540	0.729	0.143	43
QfC114	TACA	246-270	7	0.373	0.660	0.112	0.435	43
QfD102	ATCT	290-350	15	0.351	0.618	0.112	0.433	43
N8	CA	427-427	1	---	---	---	---	40
N9	TG	435-473	8	0.282	0.576	0.564	0.511	43
H8	CAA	453-453	1	---	---	---	---	43
Q1A	TTG	267-267	1	---	---	---	---	43
R9	CA	212-220	2	0.040	0.036	0.725	-0.111	43
QfD2*	TAGA	---	---	---	---	---	---	---
QfD103*	TCTA	---	---	---	---	---	---	---
QfD116*	TAGA	---	---	---	---	---	---	---
N11*	CA	---	---	---	---	---	---	---
O2*	TG	---	---	---	---	---	---	---
P5*	CAC	---	---	---	---	---	---	---

Table 3. Description of specimens analyzed including specimen, population, locality, accession number (if applicable), and collector. Voucher specimens are deposited at the University of Alabama Unionid Collection (UAUC) or the Illinois Natural History Survey (INHS).

Specimen	Population	State	Coordinates	Accession #	Collaborators
30124 (1989)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30124-1989:0056	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
30125.1 (1989)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30125-1989:0057	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
30126.8 (1989)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30126-1989:0058	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
30126.9 (1989)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30126-1989:0058	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
30128.1 (2005)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30128-1989:0060	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
SC 57 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC 94 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC 95 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC 341 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC 342 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC A031 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC A032 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC A033 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
Bou1 (2005)	Bourbeuse	Missouri	38° 21' 49.28"N 91° 10' 36.5"W	N/A	A. Roberts, S. McMurry
Bou2 (2005)	Bourbeuse	Missouri	38° 21' 49.28"N 91° 10' 36.5"W	N/A	A. Roberts, S. McMurry
BouSMO(2005)	Bourbeuse	Missouri	38° 21' 49.28"N 91° 10' 36.5"W	N/A	J.M. Serb, K.J. Roe
Saline1 (2005)	Saline	Arkansas	33.34587 N 91.98043 W	N/A	J. Harris, B. Posey, J. Seagraves
Saline2 (2005)	Saline	Arkansas	33.34587 N 91.98043 W	N/A	J. Harris, B. Posey, J. Seagraves
Saline3 (2005)	Saline	Arkansas	33.34587 N 91.98043 W	N/A	J. Harris, B. Posey, J. Seagraves
Saline4 (2005)	Saline	Arkansas	33.34587 N 91.98043 W	N/A	J. Harris, B. Posey, J. Seagraves

Table 3 (continued)

Specimen	Population	State	Coordinates	Accession #	Collaborators
Sal1 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal2 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal3 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal4 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal5 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal6 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal7 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal8 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal9 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal10 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
fragOUA1 (2002)	Ouachita	Arkansas	33° 29' 1.55"N 92° 45' 13.4"W	N/A	J. Harris, B. Posey, J. Seagraves
Oua1 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua2 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua4 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua5 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua6 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua7 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua8 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua9 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua10 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
OK1 (2005)	Little	Oklahoma	33.949203 N 94.73382 W	N/A	H. Galbraith, D. Spooner
OK2 (2005)	Little	Oklahoma	33.949203 N 94.73382 W	N/A	H. Galbraith, D. Spooner
OK3 (2005)	Little	Oklahoma	33.949203 N 94.73382 W	N/A	H. Galbraith, D. Spooner

CHAPTER 4 - GENETIC VARIATION AND POPULATION GENETIC ANALYSIS OF THE ENDANGERED WINGED-MAPLELEAF MUSSEL, *QUADRULA FRAGOSA,* USING MICROSATELLITES

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ABSTRACT

The winged mapleleaf, *Quadrula fragosa,* was declared a federally endangered species by the United States Fish and Wildlife Service in 1991 due to a drastic range reduction from multiple populations to a single population in the St. Croix River between Minnesota and Wisconsin. Since then, four additional populations of *Q. fragosa* populations have been identified in Bourbeuse River in Missouri, Saline and Ouachita Rivers in Arkansas, and Little River in Oklahoma. In order to gain an understanding of any possible genetic interaction between populations, twelve polymorphic microsatellite loci were developed and utilized to quantify genetic variation within and population structure across individuals sampled from the five available populations of the species. Results indicate a high level of genetic variation within populations, coupled with excess homozygosity indicative of high rates of within-population inbreeding. Results also indicate significant genetic divergence between populations. Nei's genetic distance (Nei, 1972) and F_{ST} tests showed that geographically proximate populations were the most closely related, and the more distant populations less so. These findings were confirmed with a Mantel test for isolation by distance. The Bourbeuse and Little populations had outlying results, though this is most likely due to the small sample size of three from each population. These results are interpreted in the context of possible conservation strategies, such as translocation and

captive breeding, under consideration by the United States Fish and Wildlife Service and the United States Army Corps of Engineers.

INTRODUCTION

Habitat degradation and the decline of freshwater mussel species in North America is indicative of a conservation crisis. North America has the highest diversity of freshwater mussels, but they are also the most endangered group of animals on the continent (Graf and Cummings, 2007; Williams *et al.,* 1993). In fact, 43% of the 297 species in North America are extinct, endangered, or threatened (Williams *et al.,* 1993). This is of great concern as freshwater mussels play a vital role in ecological systems and waterway health. Since they are filter feeders, they work to cycle nutrients in both the water and substrate. Subsequently, the decline or increase of mussels can be indicative of possible waterway contamination and/or pollution. One species that is in jeopardy is the winged-mapleleaf, *Quadrula fragosa* (Conrad 1835).

Quadrula fragosa was distributed among the Mississippi, Cumberland, Ohio, and Tennessee River drainages among twelve states (Posey *et al*., 1996). Like other freshwater mussels, *Q. fragosa* has an obligate parasitic larval stage where the glochidia must attach to the correct fish host to complete their metamorphosis to juvenile mussels (Oesch, 1984). Even though there is little information about the host fish, it is believed that the blue catfish (*Ictalurus fucatus)* and the channel catfish (*Ictalurus punctatus)* (Steingraeber *et al*., 2007, Hove *et al.,* in review) are the host fish for *Q. fragosa.* It is thus the movement patterns of these fish species that likely are the primary determinant of dispersal in *Q. fragosa*. Due to habitat degradation, however, the species was believed to have been reduced to a single

population found in the St. Croix River between Minnesota and Wisconsin (USFWS, 1991). Because of the drastic range reduction, small population size, poor recruitment, and habitat alteration in the St. Croix River between Minnesota and Wisconsin, the United States Fish and Wildlife Service classified *Q. fragosa* as federally endangered in 1991 (USFWS, 1991). Since that time, individual mussels that are morphologically similar to *Q. fragosa* have been found in the Bourbeuse River in Missouri (Andy Roberts, pers. com.), the Saline and Ouachita rivers in Arkansas (Posey *et al*., 1996), and the Little River in Oklahoma (Caryn Vaughn, pers. com.). The genetic identity of *fragosa*-like specimens has subsequently been examined and confirmed by Hemmingsen *et al.* (in preparation). In addition, Hemmingsen and Serb (in preparation) verified that *Q. fragosa* was a distinct species, not a sub-species, of *Q. quadrula* (Rafinesque, 1820). With this information on genetic identity of the southern populations, questions involving the population genetics of this species can now be addressed. In this study, we investigated the population structure and genetic diversity of *Quadrula fragosa* using variable microsatellite markers. The results of these tests will provide valuable information and improve our understanding of genetic variability within and among *Q. fragosa* populations which is critical for the development and implementation of effective conservation plans, and aid in conservation decisions by the United States Fish and Wildlife Service and the Army Corps of Engineers.

MATERIALS AND METHODS

All known populations of *Q. fragosa* were examined in this study to provide the most comprehensive coverage of genetic diversity possible (Fig. 1). Specimens were obtained from collaborators familiar with each sampling site or from museum specimens. A total of

forty-three individuals, thirteen from the St. Croix River, Minnesota/Wisconsin, three from the Bourbeuse River, Missouri, fourteen from the Saline River, Arkansas, ten from the Ouachita River, Arkansas, and three from the Little River, Oklahoma are included in the study. (Table 1).

DNA Extractions, genomic librarly enrichment, and microsatellite marker development

Given the federally endangered listing of *Quadrula fragosa,* mantle tissue was obtained from sample individuals in a non-lethal manner. All field collected tissues were stored in 95% ethanol. Tissues from the Ohio State University Museum came from preserved whole specimens collected prior to the species 1991 federal listing. For all samples, total genomic DNA extraction was performed using the spin-column protocol for animal tissues of the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, California). DNA integrity was determined using a 1% agarose gel, and concentration was determined using NanoDrop, model ND-1000 spectrophotometer. Methods for enriching the genomic library and developing microsatellite markers used Hemmingsen *et al.* (in prep).

Genotyping reactions via PCR

Individuals of each population were genotyped with 12 primer sets. Genotyping reactions were based on methods from Schuelke (2000). A fluorescent label (6-FAM) was attached to a forward primer with the M13 sequence (5'-TGT AAA ACG GCC AGT-3'). All forward primers were redesigned to contain the M13 tail at the 5' end. The forward, tailed primers were added in to the PCR reaction mix at a 1:15 ratio with the reverse and fluorescent primers. Two different *Taq* polymerases were used based on their ability to

amplify the microsatellite regions. *IDProof* (IDLabs, Inc, London, Canada) is a higher fidelity *Taq* polymerase (IDLabs, Inc) than the normal *Taq* polymerase enzyme (Weaver, 2005). The second *Taq* polymerase was in the Qiagen Multiplex PCR Master Mix (Qiagen, California), and had the highest fidelity. The Qiagen Mutliplex Mix amplified primers that did not amplify with *IDProof* and allowed weak primers to be shown with taller and more visible peaks for record and analysis.

The first PCR mix included 0.07 µl forward primer with attached M13 sequence, 1µl each of the regular reverse and M13-flourescent labeled primers, 2.5 µl 10X Buffer (with $MgCl_2$ included), 18.68 µl water, 0.5µl 10 mM dNTP's, 0.25µl *IDProof Taq* polymerase, and 1µl of 25-30 ng/µl of DNA. The PCR conditions were: 95°C for 5 minutes; 28 cycles of 94°C for 30 seconds, Optimized annealing temperature for 60 seconds, and 72°for 60 seconds. The reaction continued with 94°C for 30 seconds, 53°C for 60 seconds, and 72°C for 60 seconds. A final extension of 20 minutes at 72°C was used. A random subset was visualized on a 3% NuSieve GTG agarose gel (Lonza Group, Switzerland) to ensure the reactions worked. Then, 1.5 µl of each sample was submitted in a 96-well plate to the Iowa State University DNA Facility where they were run through the ABI 3100 Analyzer.

Primers using the Qiagen Multiplex PCR Master Mix (Qiagen, California) had the following 10 ul reaction mix: 0.07 ul forward primer with the M13 tail, 1 ul each of the reverse and M13-flourescent-labeled primers, 5 ul Qiagen Multiplex PCR Master Mix (containing *Taq,* buffer, magnesium chloride, and dNTPs), 1 ul of 20-30 ng/ul DNA, and 1.93 ul water. The PCR conditions were: 95°C for 15 minutes; 7 cycles of 94°C for 30 sec, Optimized annealing temperature for 90 sec with the temperature decreasing by 2°C each

cycle, and 72°C for 60 sec. The reaction continued with 27 cycles of 94°C for 30 sec, 50°C for 90 sec, 72°C for 60 sec., followed by a final extension at 60°C for 30 min.

Scoring of microsatellite genotypes

Genemapper (Applied Biosystems, 2005) was used to score the microsatellite alleles. Allele size in base pairs was recorded on a Microsoft Excel spreadsheet to be used in the population genetic and statistical tests. The program MICROCHECKER v.2.2.3 (Oosterhout *et al.*, 2005) was used to check for common allele scoring errors, such as stutter, large allele dropout, and null alleles. The program detects allelic frequency patterns characteristic of stuttering and null alleles. Allele frequencies were modified to compensate for null alleles within MICROCHECKER (Oosterhout *et al.,* 2005), which cannot be scored in visualization programs. Since all individuals successfully amplified for all marker loci, the Brookfield (1996) null allele estimator was used to adjust the allele frequencies. Both unadjusted and adjusted allele frequencies were utilized to determine whether the difference altered interpretation some of the statistical analyses described below.

Within-population deviations from Hardy-Weinberg

To test whether populations of *Q. fragosa* have undergone a recent bottleneck, we used the program BOTTLENECK (Cornuet and Luikart, 1996). The three models used to test for a recent bottleneck were based molecular evolution and included the Infinite Allele Model (IAM), Stepwise Mutation Model (SMM), and Two-Phase Model (TPM). Under the IAM, the number of alleles (*k*) is used to calculate the expected heterozygosity (H_e). Since the number of alleles decreases faster than the amount of gene diversity, an observed excess

of heterozygosity is indicative of a bottleneck (Cornuet and Luikart, 1996). Even though some loci follow the SMM more than the IAM, once loci begin to diverge from the SMM, they follow the IAM and the possibility of a bottleneck can be determined from there. Therefore, the TPM is used because it is an intermediate of the IAM and SMM. All five populations (St. Croix, Bourbeuse, Saline, Ouachita, and Little) were tested under all three models of evolution.

GenAlEx was used to assess whether loci were within Hardy-Weinberg equilibrium using the unadjusted allele frequencies. Deviations from Hardy-Weinberg equilibrium were determined using a Chi-square p-value for each locus across all populations and for each locus within each population. The inbreeding coefficient, F_{IS}, was also calculated using the unadjusted allele frequencies from the genotype data in GenAlEx for all polymorphic loci across all populations.

Interpopulation differentiation (AMOVA, isolation by distance)

To assess the amount and pattern of genetic differentiation among the five populations, Wright's F_{ST} and Nei's genetic distance (Nei, 1972) were calculated using GenAlEx (Peakall & Smouse, 2006) using the unadjusted allele frequencies. Genetic distances were calculated in a pairwise fashion for all twelve loci. Allelic frequency by population, allelic frequency by locus, Nei's genetic distance (Nei, 1972), and pairwise F_{st} were calculated for all polymorphic loci. Heterozygosity and polymorphism by locus and population were calculated for only the polymorphic loci (A103, A112, A130, C4, C109, C114, D102, N9, and R9) because heterozygosity is unable to be calculated for monomorphic loci. Nei's genetic distance (Nei, 1972) and values of F_{ST} for the adjusted allele frequencies

were calculated to compare with the unadjusted allele frequencies. Finally, since Nei's (Nei, 1972) and Reynolds (Reynolds *et al.,* 1983) genetic distances are based on different models, both were calculated using the unadjusted and adjusted allele frequencies to examine whether null alleles were affecting the results or not.

To examine the amount of genetic diversity using heterozygosity, we performed an Analysis of Variance (ANOVA) using Weir's method (1996) and the program SAS v.9.1 (2007). Weir's method (1996) was used because it calculated genetic diversity using only the assignment of each individual as homozygote or heterozygote. Genetic diversity was defined for this program using a binary system, 0 for homozygote and 1 for heterozygote individuals. The homozygote/heterozygote genotypes were organized by locus within individuals within populations. The test was performed using a mixed model with random variables as population, individual within population (individual (population)), locus within population (locus (population)), and individual was crossed with locus(population).

To determine the amount of molecular variance within (intrapopulation) compared to among (interpopulation) populations, an AMOVA was performed using the unadjusted allele frequencies. The AMOVA table was calculated utilizing information from all twelve codominant loci. A value for Φ_{ST} (Phi_{ST}) was calculated along with the AMOVA.

To examine isolation by distance, a Mantel test was utilized to assess the correlation of interpopulation genetic, with geographic distances. The Mantel test was conducted in GenAlEx using Nei's genetic distances (Nei, 1972) calculated from the adjusted allele frequencies, Euclidean geographic distances, and 999 permutations.

Patterns of relationship among populations

To examine and confirm population relationships, several neighbor-joining trees were created from pairwise interpopulation distances using the GenDist and Neighbor modules in Phylip v.3.67 (Felsenstein, 2007). Nei's D (Nei, 1972) and Reynold's genetic distances (Reynolds *et al.,* 1983), each of which is a different model of evolution, were calculated from both unadjusted and adjusted allele frequencies. The trees were visualized using FigTree v.1.1.1 (Rambaut, 2008) with estimates of branch support obtained via bootstrapping analysis (999 replicates) performed by Phylip's SeqBoot module.

Estimates of migration rate and effective population size

Effective migration rates and effective population sizes were estimated for each population in relation to the others. Migration rate (N_m) was calculated using the equation $N_m = ¼((1/F_{st})-1)$ (Wright, 1969). Migration rate (N_m) was calculated pairwise between populations using the equation $N_m = [((1/F_{ST})/4) \text{ X } F_{ST}]$ (Wright, 1969) in GENETIX (Belkhir, 1999).

The effective size (N_e) of each population was estimated using the online program ONeSAMP (Tallmon *et al.,* 2008) and the unadjusted allele frequencies calculated from the unadjusted genotype data. This online program combines Bayesian computation using 50,000 simulated populations with data input from the user, including the number of individuals sampled, number of polymorphic loci, and the number of repeat motif for each locus using default parameters as stated below. Since ONeSAMP (Tallmon *et al.,* 2008) uses summary statistics based on each population, an estimated census size is not necessary for the online calculation. Each population (St. Croix, Bourbeuse, Saline, Ouachita, Little) was

submitted separately specifying the number of individuals sampled (13, 3, 14, 10, 3, respectively), number of polymorphic loci (8, 7, 8, 9, 7, respectively).

RESULTS

Even though 18 primer sets were designed, only 12 were used for the population genetics analysis. Of the six that were not used, three (D2, D116, and P5) were not included due to inconsistency in peak calling due to stutter. The other three (D103, N11, and O2) were not used due to inconsistency or inability to amplify the region of interest. The remaining twelve loci were used in the population genetic tests described herein.

Large allele dropout was not detected in any of the loci in any of the populations examined using MICROCHECKER (Oosterhout *et al.*, 2005). The Bourbeuse and Little populations were not included in this analysis because of their small sample size of three. Stuttering was detected in loci A130 and C114 in the St. Croix population, in locus A112 in the Saline population, and at locus C114 in the Ouachita population. Adjusted allele frequency effects were focused on those loci that displayed patterns characteristic of null alleles. Null alleles were detected in loci A130, C4, C114, and D102 in the St. Croix populations, in loci A112, A130, C4, C114, and N9 in the Saline population, and in loci A112, A130, C4, C114, D102, and N9 in the Ouachita population. Null alleles cannot be detected visually because they appear to be missing data at the locus, most likely due to a mutation in the primer binding site not allowing amplification of the desired region. In order to obtain the most accurate conclusions from the population genetic tests, the adjusted allele frequencies were compared to the unadjusted allele frequencies in each population genetic test and analysis.

Table 2 shows the characteristics of all 12 pursued and 6 non-amplifiable primers, including sequence, annealing temperature, and repeat motif. Table 3 describes each primer's repeat motif, repeat size range, the number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), significance, and inbreeding coefficient (F_{IS}) values.

Allele frequency adjustments

In the St. Croix population, stuttering was predicted in loci A130 and C114. Null alleles were predicted in loci A130, C4, C114, and D102. Evidence of stuttering was detected in the Saline population at locus A112. Null alleles were detected in loci A112, A130, C4, C114, and N9. The Ouachita population had locus C114 detected for patterns of stuttering. Loci A112, A130, C4, C114, D102, and N9 were detected for having null alleles. Since the Little River, Oklahoma and Bourbeuse River, Missouri populations only sampled three individuals each, there was not enough data for MICROCHECKER to perform the analysis. Alleles exhibiting stutter patterns and null alleles were included in all analysis. Adjusted allele frequencies were calculated in MICROCHECKER to correct for the detecting null alleles.

Within-population deviations from Hardy-Weinberg

Analysis of the bottleneck test indicated that there was no evidence of a bottleneck for the St. Croix, Saline, and Ouachita populations. A recent bottleneck was detected in the Bourbeuse and Little populations (n = 3). F_{IS} values with the unadjusted allelic frequency calculations ranged from -0.500 in loci A103, A130, C114, and N9 in the Little population to 1.000 in loci A112, C114, and D102 in the Bourbeuse population, as well at locus N9 in the

Saline population, and locus C109 in the Little population. When examined across all populations, the F_{IS} values ranged from -0.421 in locus A103 to 0.511 in locus N9. The observed (H_o) and expected (H_e) heterozygosities for each locus across all populations are shown in Table 3. Locus R9 had the lowest H_o at 0.040, and locus C4 had the highest H_o at 0.692. The lowest H_e was at locus R9 at 0.036, and the highest H_e was at C4 at 0.838. Even though none of the loci showed significant deviations from Hardy-Weinberg equilibrium across all populations, some individuals showed deviations at some loci. For example, the St. Croix population had a p-value of 0.000 for locus A130, 0.039 for locus C4, 0.001 for locus C109, 0.013 for C114, and 0.034 for locus N9, all indicating a significant ($\acute{\alpha} \leq 0.05$) deviation. Significant p-values for the Saline population were 0.000 for locus A112, 0.004 for locus C4, 0.000 for C114, and 0.000 for N9. The Ouachita population had significant deviations of 0.000 for locus A130, 0.031 for locus C4, 0.011 for locus C114, 0.043 for locus D102, and 0.003 for locus N9. The Bourbeuse and Little River populations did not show any significant deviation from Hardy-Weinberg equilibrium for any locus.

Interpopulation differentiation (AMOVA, isolation by distance)

The pairwise F_{ST} table (Table 4), using the unadjusted allele frequencies, showed that the Saline and Ouachita populations had the lowest pairwise F_{ST} value of 0.058. The Bourbeuse River, Missouri and Little River, Oklahoma populations have the highest pairwise F_{ST} value of 0.193 each.

Nei's (Table 5) and Reynolds genetic distances were calculated for the unadjusted and adjusted allele frequencies. Using the unadjusted allele frequencies, Nei's D showed that the Little and Bourbeuse populations has the greatest amount of genetic distance at 0.332. The Ouachita and Saline populations showed the least amount of genetic distance at 0.112.

The Reynolds distance showed the same pattern with the Saline and Ouachita populations with a distance of 0.125, and the Little and Bourbeuse populations with a distance of 0.275. Using the adjusted allele frequencies, Nei's D showed the same trend as with the unadjusted allele frequency calculations. The Nei's D for the Little and Bourbeuse populations was 0.240, showing the most amount of genetic distance. The least amount of genetic distance was between the Ouachita and Saline populations (0.083). The Reynolds distance, again, showed the same trend with the Ouachita and Saline populations with a distance of 0.074, and the Little and Bourbeuse populations with a distance of 0.332.

Table 6 describes the results of the ANOVA. The p-values at the level of the locus was 0.0117, population was 0.7562, individual (population) was 0.3474, and locus (population) was 0.0069. These values indicated that the highest amount of genetic diversity occurs at the locus level within populations.

There was a significant difference (Φ_{st}= 0.12, p = 0.001) between the among population variation (12%) and the within population variation (88%; Fig. 2, Table 7).

Results from the Mantel Test for isolation by distance showed that the St. Croix and Saline were the most distant, where the Saline and Ouachita populations were the most closely related (Fig. 3 and Table 8). A trend of regression was unable to be calculated because there were only five populations, which were not sufficient to assess significance in a Mantel test.

Patterns of relationship among populations

The neighbor-joining trees at the level of unadjusted and adjusted allele frequencies had differing topologies, but when comparing the trees at the level of Nei's (Nei, 1972) and

Reynolds (Reynolds *et al.,* 1983) genetic distances, the topologies were the same (Fig. 4). Therefore, trees using Nei's genetic distance (Nei, 1972) will be described for each calculated allele frequency.

Using the unadjusted allele frequencies, the relationship of the St. Croix and Bourbeuse populations, and the relationship of the Little and Ouachita populations are well supported with bootstrap values of 1.00 (Fig. 4b). The St. Croix/Bourbeuse relationship with the Saline population is supported with a value of 0.591. Support for the Little/Ouachita relationship with the Saline population is 0.49.

The neighbor-joining tree, using the adjusted allele frequencies, shows that the relationship of the St. Croix and Bourbeuse populations, and the relationship of the Saline and Ouachita populations are both well supported with bootstrap values of 1.00 (Fig. 4a). Support for the St. Croix/Bourbeuse relationship with the Little River population is 0.578. The relationship for the Saline/Ouachita clade and the Little River population was supported with a value of 0.678.

Relative and estimated effective population sizes

Calculations based on F_{ST} values resulted in N_m values ranging from 1.41 individuals between the St. Croix and Little populations to 9.64 individuals between the Bourbeuse and Ouachita populations (avg. = 3.43) (Table 9). Estimates of N_e from the ONeSAMP included the mean and median suggested effective population sizes, as well as upper and lower 95% confidence intervals (Table 10). The estimated mean effective population size for St. Croix is 22, the Saline is 16, the Ouachita is 16, and the Little is three. The test was unable to be

completed for the Bourbeuse population due to the excess of missing data for a locus. Only one individual of three did not show an allele for locus A112.

DISCUSSION

This study sought to examine how genetic diversity is distributed across *Quadrula fragosa,* and specifically determining genetic variation within or between the known five populations using microsatellites. Results from this study indicate that the populations exhibit a high degree of genetic distance, little to no gene flow between populations, and that most of the genetic variation of the species is found within each *Q. fragosa* population. Approximately 88% of the genetic variation within the *Quadrula fragosa* individuals sampled is found within the populations, as determined by the AMOVA. Other mussel species exhibit a similar genetic variation distribution. For example, a population genetic study examining *Amblema plicata,* a freshwater mussel found in the central United States, had a similar amount of genetic variation, 82% (Elderkin *et al.,* 2007), as *Q. fragosa.* Another unionid, *Epioblasma torulosa,* showed the same pattern with 91.83% of the variation found within populations (Zanatta and Murphy, 2007). Since the mussels are sessile and separated by hundreds of river miles, most likely not reproducing with other populations, one would expect to find more variation occurring within the population instead of outside it. Results from the AMOVA, test of genetic differentiation (F_{ST}), ANOVA, and calculation of the effective rate of migration (N_m) support the geographical separation of the population. This separation also indicates that there is little to no gene flow between populations.

Since a higher degree of genetic variation is found within populations, the amount of genetic differentiation (F_{ST}) should increase as populations become further apart. For example, since the Saline and Ouachita populations share a river confluence, we would expect a lower F_{ST} value compared to the St. Croix and Little populations which do not share any river confluence. Indeed, this was found with the Saline and Ouachita populations, and the St. Croix and Little populations. The St. Croix River between Minnesota and Wisconsin is a tributary to the upper Mississippi River that flows into the Gulf of Mexico, but the Little River in Oklahoma is tributary to the Red River which flows into the Mississippi River and into the Gulf of Mexico. Therefore, the two populations do not have a current way to exchange alleles downstream of each other, increasing the amount of population differentiation. Data from the previous studies on freshwater mussels indicate a similar range of F_{ST} values of 0.054-0.151 (moderate genetic differentiation) to those from this study. Elderkin *et al.* (2007) described F_{ST} values ranging from -0.021-0.252 (little to very great genetic differentiation) for *Amblema plicata,* a commonly found mussel suggested to be a model species for freshwater mussel population genetics because of its intact genetic structure. Zanatta and Murphy (2007) described pairwise F_{ST} values ranging from 0.019-0.126 (little to moderate genetic differentiation) for *Epioblasma torulosa,* an endangered mussel historically found near *Q. fragosa* but now only found in Pennsylvania and Ontario, Canada. Values of F_{ST} fall within the range of *A. plicata* and slightly above the maximum for *E. torulosa*. This indicates that *Q. fragosa* is on the border between a model mussel species and another endangered mussel species, and displays a high amount of population genetic structure.

Results of the test of isolation by distance, with Nei's genetic distance (Nei, 1972) versus geographical distance in river miles resulted in a clustering pattern showing the expected results for increasing geographic distance, but were not significant with the increase in genetic distance. For example, the northern St. Croix population is the most geographically distant population sampled in this study, however, the genetic distance is smaller (instead of larger) with the southern Bourbeuse, Saline, Ouachita, and Little populations. The results of the Mantel test for isolation by distance did not give a significant conclusion for the linear pattern, but this could be due to small and uneven sample sizes. Another reason that the test is not significant could be due to the inability to detect genetic distances based on geographic separation. If the populations were separated by dam implementation relatively recently, then the detected genetic distance would not be due to an increase in geographic distance, but by a drastic limitation or halt in gene flow.

Conflicting results arose when allele frequencies were used to calculate genetic distances visualized in a neighbor-joining tree. Comparing Nei's (Nei, 1972) and Reynolds (Reynolds *et al.,* 1983) genetic distances showed no difference in topology, meaning that the respective models did not alter the relationships of the populations. However, tree topology was different when comparing the unadjusted versus adjusted allele frequencies. While the unadjusted allele frequency tree was unexpected based on geography with the Ouachita River, Arkansas population more closely related to the Little River, Oklahoma population than the Saline River, Arkansas, which share a river drainage, it was poorly supported. The adjusted allele frequency tree showed stronger support for the geographically correct relationships, but it was still quite weak with bootstrap support. The most likely reason for

the discrepancy is the difficulty in accounting for null alleles and the small, unequal sample sizes among the populations.

Knowing that genetic variation is highest within populations and that the populations are well differentiated, one can begin to speculate the direction of gene flow and migration. This study attempted to calculate a value of N_m, the effective migration rate, between each population (Table 9). These values should be taken with caution, however, because they are based on the equation $F_{ST} \sim 1/ (4N_m +1)$, and assumptions about population sizes by Wright (1931). Wright (1931) determined that one migrant per generation is sufficient to maintain the amount of genetic variation within a population. Even though this study calculated effective rates of migration well above one migrant per generation, this model is based on assumptions that may not apply to *Quadrula fragosa.* Factors including a small population size, the knowledge that genetic drift occurs at an increased rate in small populations, and uneven population sizes could explain why Wright's (1931) model would not apply to *Q. fragosa* (Storfer, 1999), as the species is endangered (small populations size), has uneven population sizes, and no record of observed migrants between populations. However, if *Q. fragosa* is truly encountering the calculated number of migrants per generation, then the population is experiencing, or historically experienced a decrease in genetic drift due to the increase of "new" alleles incorporated into the population. These estimates are important to the USFWS and USACoE for possible translocation and captive breeding programs.

Not only has the construction of dams made it more difficult for migrants to move, they have also further segregated populations of mussels. The dams altered the habitat and dispersal, and I believe they have also had an effect on fish host movement. *Quadrula fragosa,* like most freshwater mussels, requires a fish host for its larvae (glochidia) to mature.

One scenario for the high amount of inbreeding calculated in this study may result from the limited mobility of host fish. If the fish do not swim very far during glochidial metamorphosis, it is possible that the juveniles will drop in a similar, or even the same, location as their parents and siblings, increasing inbreeding rates, F_{IS}. The identified host fishes (Hove, 2004) of *Q. fragosa, Ictalurus punctatus* (channel catfish) and *I. furcatus* (blue catfish) both spawn during the summer (Smith, 1979). If a spawning fish were to be infected by *Q. fragosa* glochidia during the mussels' short brooding season in early autumn (Heath *et al.,* 2000), the *Q. fragosa* glochidia could transform into juvenile mussels over the winter. Though the fish may have winter and spring to travel, it must travel hundreds of miles and through a minimum of 29 dams to reach the lower Mississippi River then return north to spawn again. This restricted movement may result in mature juveniles dropping too close to the parental population to keep a high level of genetic variation, therefore continuing a high rate of inbreeding. Additionally, since *Q. fragosa* is an endangered species, it is possible that there may not be many females brooding and releasing glochidia. If a fish were infected by the glochidia by only one female, then the juveniles that matured and dropped from the fish would be full-siblings in a close proximity.

In addition to impeding mussel "movement," the many dams along the rivers impede movements of host fish, which could hinder the dispersal of mussel glochidia to more distant locations. Zigler *et al.* (2004) performed a study examining how the movements of paddlefish were impeded due to the dams on the upper Mississippi. They found that fish had more difficulty traveling through the dams both swimming upstream and downstream, but due to the increased water velocity coming from the dams, the fish had a much more difficult traveling upstream to spawn (Zigler *et al.,* 2004). If dams impede catfish movements in a

similar fashion as the paddlefish, it could be a partial explanation for the decrease in migration and gene flow, and an increase in the inbreeding rate of the populations of *Quadrula fragosa* we studied. Even though individuals of *Q. fragosa* are long-lived, effects of inbreeding will still occur but will not be observed until generations and possibly hundreds of years later, making it too late to effectively preserve the species.

Another historical pressure affecting *Quadrula fragosa* is industrial and agricultural practices. It is known that industries and agricultural farms disposed of waste into nearby river systems. This influx of fertilizers and liquid waste negatively affected mussel habitat by changing water pH and introducing toxic substances. Even though industries have adopted cleaner disposal practices, runoff from farms remain an area of concern for ecologists and conservation managers. What if *Q. fragosa* was one homogenous population that extended all along the Mississippi River and its tributaries, but due to agricultural runoff, populations were extirpated leaving isolated populations of the species isolated in the upper tributaries? Since the one population was divided into five isolated populations, the rate of gene flow would be considerably reduced and the rate of genetic drift increased due to the lower amount of genetic varation.

Discovering that the majority of genetic variation was found within populations, instead of between populations, this study further examined the amount of variation at the population level. Using a binary system of heterozygosity, a value of 0 for homozygous individuals and 1 for heterozygous individuals, we could assess the level within the population that genetic diversity was most prevalent. The significant result of the ANOVA showed that the locus has the biggest effect on genetic variation. Therefore, to increase the amount of genetic diversity, we must work to increase the diversity at the locus level within

each population separately. This could be implemented by genotyping individuals for a particular locus, or loci, to ensure that it would introduce new alleles into the population of interest.

When considering species rehabilitation, it is important to consider the amount of breeding individuals, N_e, within each population. This is important because a population can be large, but if there are few breeding individuals, then rates of inbreeding and genetic drift can increase and cause population decline. Even though, ONeSAMP (Tallmon *et al.*, 1998) provided estimates for effective population size, one must hesitate before accepting these numbers at face value. Factors including small and unequal sample sizes, the recent release of this program, and the small amount of knowledge regarding the census sizes may change the effective population sizes in future studies. This number is valuable for assessing the future reproducibility of each population, as well as the possibility for translocating individuals to other populations. Since *Quadrula fragosa* is an endangered species, it is difficult to obtain an accurate representation of each population. It was unexpected to find that the effective population size for the Little River population in Oklahoma was three because the sample size was also three, but this result may be due to the small sample. The estimated N_e was a greater value than the other sample sizes, excluding the Bourbeuse population, but it raises the question of how that compares to the census population size. For example, if the estimated N_e for the St. Croix River population is 22, but the last census size was approximately 24 (Hornbach *et al.*, 1996), removing 22 individuals would decimate the donor population. Also, a small effective population size does not necessarily mean that a new population can be started with that amount of individuals because they may not contain enough genetic variation to continue the new population. Zanatta and Murphy (2007)

attempted to examine the effective population size for *Epioblasma torulosa rangiana,* but due to incomplete knowledge of microsatellite mutation rates in bivalves, found that the estimated effective population size did not correlate with the measured population estimates. Census sizes for the Saline and Ouachita populations have a large and inconsistent spread. Harris (2006) estimated *Q. fragosa* population sizes to range between 510 $\pm$ 253 and 9,217 $\pm$ 4,114 individuals per sample site in the Saline River, Arkansas, and between 0 and 1,770 $\pm$ 1,227 individuals per sample site in the Ouachita River, Arkansas.

The results showed no significant deviation from Hardy-Weinberg equilibrium for all loci across all populations. However, this masks the deviations found at the population level. The St. Croix, Saline, and Ouachita populations all exhibited significant deviations at four to five loci, but since there are no overlying trends with the loci within each population, the differences in significance are most likely due to scoring error or small sampling size. Reaching a tangible conclusion for deviations in Hardy-Weinberg equilibrium will be difficult to obtain by increasing sample size due to the species federal endangered listing.

Using excess heterozygosity to suggest whether the five populations encountered a recent bottleneck, we concluded that the Bourbeuse and Little populations have encountered a recent bottleneck. Even though this conclusion was expected due to the number of individuals sampled from each location, it is worthwhile to increase sampling and re-examine the Little population (due to the lack of individuals from the Bourbeuse, however, increased sampling is not possible). Cornuet and Luikart (1996) suggest that a minimum of 20 sampled individuals is needed to achieve a high power of analysis. They also suggest that if increasing the number of loci sampled will increase the power more than the number of individuals sampled (Cornuet and Luikart, 1996). Therefore, to obtain a more exact

estimation of if/when the populations encountered a bottleneck, more loci as well as more individuals need to be sampled.

The negative F_{IS} values found in loci A103 and R9 indicate an excess of heterozygosity. Locus A103 only displayed two allele types (298 and 302) in all individuals in all populations, and the combinations of these two allele types resulted in a greater amount of heterozygosity than expected. Locus R9 was homozygous at allele 220 for all individuals in all populations except for one individual in the Ouachita population. This one anomaly resulted in the -0.111 F_{IS} value for locus R9, so it cannot be interpreted that the Ouachita population exhibits excess heterozygosity at locus R9. Loci C4 and C109 displayed F_{IS} values of 0.174 and 0.143, respectively, which is similar to the value expected for half-sibling inbreeding, 0.125. Locus A130 displayed an F_{IS} value of 0.321, which exceeds the value expected for full-sibling inbreeding, 0.25. Finally, loci A112, C114, D102, and N9 displayed F_{IS} values of 0.5, 0.435, 0.433, and 0.511, respectively, which are similar to the value expected for self-inbreeding, 0.5. When the F_{IS} values for all loci across all populations were averaged, the value was still indicative of full-sibling inbreeding, 0.220. Compared to Geist and Kuehn's study (2005), our value is much higher, 0.220 versus 0.079. However, the study performed by Geist and Kuehn (2005) included a mussel species from a different family of freshwater mussels, and more than five populations spread across five river drainages. Our study was on a smaller scale with fewer populations and individuals (43 individuals from five populations versus 558 individuals from 24 populations; Geist & Kuehn, 2005). Our study's numbers are not far from expected when considering the reproductive biology and host fish dependency of mussels described earlier. Another consideration when examining rates of inbreeding and reproductive biology is the possibility

of hermaphroditism. Freshwater mussels of some species, such as *Margaritifera margaritifera,* exhibit hermaphroditism if mussel populations decrease to a low density (Bauer, 1987). Garner *et al.* (1999) found that a small portion of the population (2%), of *Q. metanevra* in Tennessee was monoecious. Therefore, if *Q. fragosa* were to reveal hermaphroditic individuals in times of low density, it would be an explanation for the high rates of inbreeding observed. A study (Dupont *et al.,* 2007) examining population genetics and inbreeding coefficients in gastropods, which are known to exhibit hermaphroditism, showed values ranging from 0.05-0.15 for *Crepidula fornicata* L. Unfortunately, these do not help to explain the effect of hermaphroditism on inbreeding coefficients because these values are lower than the majority of the present study's observations. One possibility in the inability to formulate an overall conclusion may be due to the fact that Dupont *et al.* (2007) looked at F_{IS} values across five microsatellite loci, whereas we examined each locus separately.

Much work still needs to be completed to fully understand the population structure and interactions of *Quadrula fragosa.* Information on life history traits, especially reproduction, and interaction with host fish needs to be explored to identify if/how this species of mussel is dispersed. Having a more in-depth knowledge of the biology of *Q. fragosa* and host fish-mussel interactions will allow conservation agencies like the United States Fish and Wildlife Service and United States Army Corps of Engineers to make the most well-informed decisions for species conservation programs. For example, if translocation programs, like re-introduction, are to be implemented, then there needs to be a comprehensive understanding of the genetic differentiation between the individuals to be moved and the individuals currently residing in the target location. This project will also

serve as an example of the development and implementation of microsatellites for other freshwater mussel species. With little knowledge about the system and the rapid decrease of freshwater mussels in the United States, a method to quickly assess the conservation status of threatened and endangered species will be important for their conservation.

This study suggests that translocation programs should not be implemented in the near future. Far too little is known about the habitat specifics and life history traits to securely begin new populations in the historical distribution of *Q. fragosa.* The possibility of local adaptations needs to be considered as well. Since the five populations have a high degree of genetic distance, it is possible that the northern St. Croix population has adapted to colder water temperatures while the Saline, Ouachita, and Little populations have adapted to warmer water temperatures. Juvenile mussel development and metamorphosis is temperature dependent, and the difference of water temperatures could have an effect on the reproductive periods and synchronization of adult mussels. Steingraeber *et al.* (2007) determined that *Q. fragosa* juveniles develop at a faster rate in warmer waters than in cooler waters. Before translocating individuals, one must also consider how the removal of individuals will affect the donor population, the recipient population, and/or the augmentation of a new population. Will removing individuals cause an increased rate of inbreeding due to the removal of variable alleles? Will the recipient population experience outbreeding depression, washing out any local variation the population may have? Will a new population experience an increased level of inbreeding due to the founder effect?

A more feasible option is the implementation of captive breeding programs. If mussels could be reared in an artificial, yet similar, lab environment, offspring could be introduced back into the population to increase population size, or could be introduced into

other populations in hopes of increasing the amount of genetic diversity. Like translocation programs, though, one must be wary of creating an outbreeding depression in the augmented population. If a captive reared population were to become robust enough, they could begin to be introduced into the historical distributions, provided that the habitat is once again suitable for the species. Based on our data, the Ouachita and Saline populations would be the most appropriate populations to mix. They share a river confluence, inhabit the same geographical area of Arkansas, and have similar aquatic environments. These populations are also two of the more robust populations (with a higher amount of genetic variation) that could be used to increase the amount of variation in the St. Croix population or for captive breeding programs for reintroduction into localities of the historic *Q. fragosa* distribution.

Finally, if a successful rehabilitation program is to take place with *Quadrula fragosa,* the public must be involved in its assessing the success of the population(s), and made aware of the dire need to conserve this species. Other successful rehabilitation programs in the US have included the American bald eagle, the California condor, and the timberwolf. These species were made into icons for preservation, and the public responded by improving habitat, decreasing poaching, repealing use of hazardous chemicals, etc. To prevent *Q. fragosa* from joining the approximate 50% of freshwater bivalves that are already extinct or in danger of becoming extinct (Bogan, 1993), the public must be made aware that *Q. fragosa* is a special type of freshwater "clam," and that its existence is vital to the health of their waterways and persistence of biodiversity.

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Figure Legends

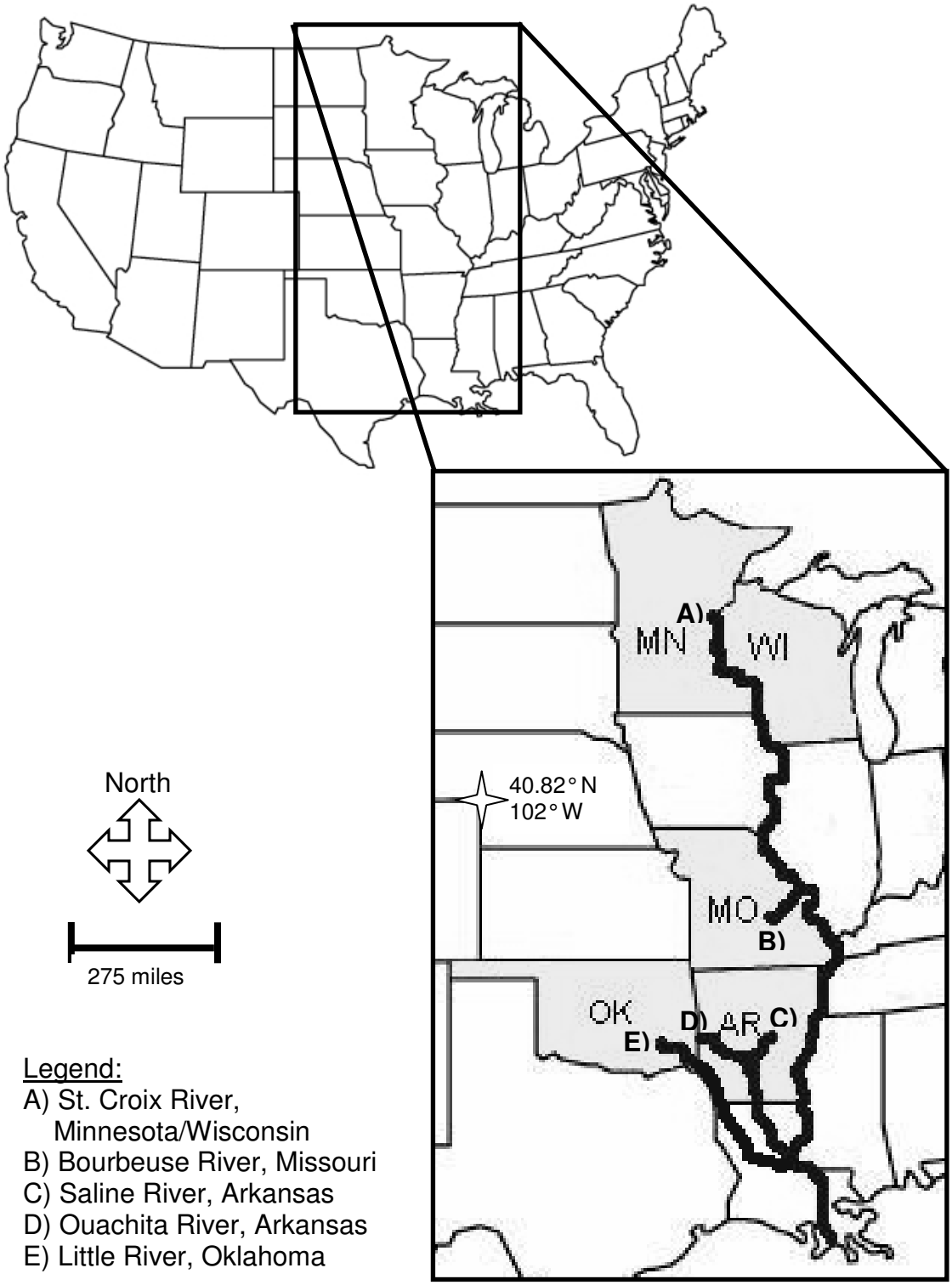
Figure 1. Map of specimen localities and adjacent river systems. Location A) denotes the population found in the St. Croix River between Minnesota and Wisconsin, B) shows the Bourbeuse River population in Missouri, C) the Saline River population in Arkansas, D) the Ouachita River population in Arkansas, and E) the Little River population in Oklahoma.

Figure 2. Results of the AMOVA displayed as a pie-chart, 12% of molecular variance is found among populations, and 88% is found within populations.

Figure 3. Graphical view of the Mantel test using data from Table 8, including populations from the St. Croix River, Minnesota/Wisconsin (SC), Bourbeuse River, Missouri (Bou), Saline River, Arkansas (Sal), Ouachita River, Arkansas (Oua), and Little River, Oklahoma (Lit).

Figure 4. Neighbor-joining tree of populations using genetic distances. Panel a) shows relationships based on unadjusted allele frequencies and Nei's genetic distance. Panel b) shows relationships based on adjusted allele frequencies and Nei's genetic distance. Boostrapping was used to account for the differences in sample sizes in each population, and values are based on 999 replications and displayed above the branches.

Figure 1.



Legend:
A) St. Croix River, Minnesota/Wisconsin
B) Bourbeuse River, Missouri
C) Saline River, Arkansas
D) Ouachita River, Arkansas
E) Little River, Oklahoma

Table 1. Collected specimens with population, locality, accession number and collector. Museum specimens were from the Ohio State University Museum.

Specimen	Population	State	Coordinates	Accession #	Collaborators
30124 (1989)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30124-1989:0056	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
30125.1 (1989)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30125-1989:0057	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
30126.8 (1989)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30126-1989:0058	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
30126.9 (1989)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30126-1989:0058	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
30128.1 (2005)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	30128-1989:0060	D.J. Heath, G.A. Miller, A.R. Weisbord, S. Placzek
SC 57 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC 94 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC 95 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC 341 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC 342 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC A031 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC A032 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
SC A033 (2007)	St. Croix	Minnesota/Wisconsin	45° 25' 39.03"N 92° 37' 29.38"W	N/A	N. Rowse, P. Delphey
Bou1 (2005)	Bourbeuse	Missouri	38° 21' 49.28"N 91° 10' 36.5"W	N/A	A. Roberts, S. McMurry
Bou2 (2005)	Bourbeuse	Missouri	38° 21' 49.28"N 91° 10' 36.5"W	N/A	A. Roberts, S. McMurry
BouSMO(2005)	Bourbeuse	Missouri	38° 21' 49.28"N 91° 10' 36.5"W	N/A	J.M. Serb, K.J. Roe
Saline1 (2005)	Saline	Arkansas	33.34587 N 91.98043 W	N/A	J. Harris, B. Posey, J. Seagraves
Saline2 (2005)	Saline	Arkansas	33.34587 N 91.98043 W	N/A	J. Harris, B. Posey, J. Seagraves
Saline3 (2005)	Saline	Arkansas	33.34587 N 91.98043 W	N/A	J. Harris, B. Posey, J. Seagraves
Saline4 (2005)	Saline	Arkansas	33.34587 N 91.98043 W	N/A	J. Harris, B. Posey, J. Seagraves

Table 1 (continued)

Specimen	Population	State	Coordinates	Accession #	Collaborators
Sal1 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal2 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal3 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal4 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal5 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal6 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal7 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal8 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal9 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
Sal10 (2007)	Saline	Arkansas	33.32668 N 91.97578 W	N/A	Chris Davidson-USFWS
fragOUA1 (2002)	Ouachita	Arkansas	33° 29' 1.55"N 92° 45' 13.4"W	N/A	J. Harris, B. Posey, J. Seagraves
Oua1 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua2 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua4 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua5 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua6 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua7 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua8 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua9 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
Oua10 (2007)	Ouachita	Arkansas	33.67001 N 92.86965 W	N/A	John Harris-AR State Univ
OK1 (2005)	Little	Oklahoma	33.949203 N 94.73382 W	N/A	H. Galbraith, D. Spooner
OK2 (2005)	Little	Oklahoma	33.949203 N 94.73382 W	N/A	H. Galbraith, D. Spooner
OK3 (2005)	Little	Oklahoma	33.949203 N 94.73382 W	N/A	H. Galbraith, D. Spooner

Table 2. Characteristics of 18 microsatellite loci. Fluorescently labeled primers (6-FAM) are in bold, the annealing temperature (°C), and repeat motif. *Primers not used due to poor PCR amplification.

Name	Primer Sequence	Anneal Temp	Repeat Motif
QfA103	**F-5'-GCA CAC CTT ATT CAT TTG AGA-3'**	49	CA
	R-5'-AAT GTC TTC CCC ATG ACT AAA-3'		
QfA112	**F-5'-ACT TGC TCC AAA ACT TGT AGA G-3'**	56	CA
	R-5'-GGA ATG GTT CAG ACT ATG ACC-3'		
QfA130	**F-5'-TGA GAA ATC GTG ATG ACT CAG-3'**	58	TG
	R-5'-CCT ACC TAC CTT CAT GTG GTC-3'		
QfC4	**F-5'-TGT CCT TCT CTG TGA ATG TTT G-3'**	58	TACA
	R-5'-GCA CTC CAT AAA TGC AGG TAA T-3'		
QfC109	**F-5'-GAC AGG AAA TAA AGG GTG TC-3'**	55	TATG
	R-5'-GCA ATG TAA TAT GGT ATG CAC-3'		
QfC114	**F-5'-TCC ATG TTT TTC TCC TCC TCT A-3'**	58	TACA
	R-5'-CAC CCT TGC TTA TAG CGT AGT C-3'		
QfD102	**F-5'-TGG ACA ATT CAT CAA GTC AAG-3'**	53	ATCT
	R-5'-CTT TGT TTT CCA AAC CAT ACA G-3'		
N8	**F-5'-AGC TTG GGA TCA CCT ATG ACC C-3'**	63	CA
	R-5'-GCC CTT CAG ACA GTG TCC TCT CTG CT-3'		
N9	**F-5'-TCG TCT ACC ACC TCT GCA ACA CAT ACC G-3'**	68	TG
	R-5'-GGC AGA GAG GTC ACA ACC CCG GA-3'		
H8	**F-5'-ACC CTT GTG GGT GTG GTG TGG AGA ACG-3'**	68	CAA
	R-5'-GGA TCC AAT CGG AGA GCC TGA GGT-3'		
Q1A	**F-5'-ACA GTT CTA GTG TCC GAG GAG TCA CTG G-3'**	64	TTG
	R-5'-GGT GTA TTG TGT CAT CGG TGC TGC CA-3'		
R9	**F-5'-AGC TTG GGA TCG GAG TTG CAG CCA GC-3'**	66	CA
	R-5'-GGA CAC CCC AGT GTG TAA GAA CA-3'		
QfD2*	**F-5'-TGG ATG TTA TTG TGC TTA ACG A-3'**	50	TAGA
	R-5'-GCC ATT TAT CAA AGA ATG CAG-3'		
QfD103*	**F-5'-ACG TGT AAC CGA TTG GTA TAT C-3'**	55	TCTA
	R-5'-GTA TGA AGG GAC GAA AAT GTA C-3'		
QfD116*	**F-5'-CCA TGT AAA GGT TTG CAT TAA C-3'**	48	TAGA
	R-5'-TGG ACA CAC CAC ATA TAC AGA C-3'		
N11*	**F-5'-TGT GGC TGT GCT GGT GAC TCA TTT CC-3'**	68	CA
	R-5'-CCA TGC CAT CAG GTG CAG GA-3'		
O2*	**F-5'-AGC AGA CTT CAT CGA GAC AAA AAT GGT CGG-3'**	66	TG
	R-5'-CCA GTT CAT CAG TCG GTA TAT TCT TCC GCT-3'		
P5*	**F-5'-TCG CCA CGG TAC AAT CAG TTC TTG CAA CG-3'**	62	CAC
	R-5'-GCG TGT CTG ACG AGC AAT AGG T-3'		

Table 3. Results of microsatellite screen. Primers are listed with repeat motif, size range, number of alleles (A), observed and expected heterozygosities (H_O and H_E), p-value based on chi-square, and number sampled (n). *Primers not used due to poor PCR amplification.

Name	Repeat Motif	Size Range (bp)	A	H_o	H_e	p	F_{IS}	n
QfA103	CA	298-302	2	0.484	0.341	0.184	-0.421	43
QfA112	CA	160-180	10	0.327	0.654	0.157	0.500	42
QfA130	TG	287-323	14	0.489	0.720	0.423	0.321	43
QfC4	TACA	236-380	28	0.692	0.838	0.451	0.174	43
QfC109	TATG	177-285	10	0.463	0.540	0.729	0.143	43
QfC114	TACA	246-270	7	0.373	0.660	0.112	0.435	43
QfD102	ATCT	290-350	15	0.351	0.618	0.112	0.433	43
N8	CA	427-427	1	---	---	---	---	40
N9	TG	435-473	8	0.282	0.576	0.564	0.511	43
H8	CAA	453-453	1	---	---	---	---	43
Q1A	TTG	267-267	1	---	---	---	---	43
R9	CA	212-220	2	0.040	0.036	0.725	-0.111	43
QfD2*	TAGA	---	---	---	---	---	---	---
QfD103*	TCTA	---	---	---	---	---	---	---
QfD116*	TAGA	---	---	---	---	---	---	---
N11*	CA	---	---	---	---	---	---	---
O2*	TG	---	---	---	---	---	---	---
P5*	CAC	---	---	---	---	---	---	---

Table 4. Representation of the amount of genetic differentiation among populations. Values below the diagonal are pairwise F_{ST} values and those above are the corresponding significance values. Bolded values indicate a significance of $p < 0.05$. *Values indicate populations with the greatest and least amount of genetic differentiation.

	St. Croix	Bourbeuse	Saline	Ouachita	Little
St. Croix		0.0750	**0.0050**	**0.0050**	**0.0050**
Bourbeuse	0.0610		**0.0450**	0.1000	0.1100
Saline	**0.0888**	**0.0597**		**0.0050**	**0.0250**
Ouachita	**0.1276**	0.0253	**0.054***		0.0550
Little	**0.151***	0.1282	**0.0943**	0.0849	

Table 5. Comparison of Nei's genetic distance (D) using the unadjusted and adjusted allele frequencies.

a) Nei's genetic distance calculated using the unadjusted allele frequencies.

Nei Genetic Distance	Genotypes	Pairwise Population Matrix of Nei Genetic Distance			
St. Croix	Bourbeuse	Saline	Ouachita	Little	
0.000					St. Croix
0.186	0.000				Bourbeuse
0.152	0.205	0.000			Saline
0.215	0.177	0.125	0.000		Ouachita
0.263	0.275	0.212	0.205	0.000	Little

b) Nei's genetic distance calculated using the adjusted allele frequencies specified by MICROCHECKER.

Nei's Genetic Distance via Phylip-GenDist for Adjusted Allele Frequencies					
St. Croix	Bourbeuse	Saline	Ouachita	Little	
0.000					St. Croix
0.195	0.000				Bourbeuse
0.164	0.228	0.000			Saline
0.185	0.187	0.083	0.000		Ouachita
0.265	0.274	0.240	0.222	0.000	Little

Table 6. Summary of ANOVA table using Weir's (1996) method of detecting genetic variation using heterozygosity, describing the source (variable used in the analysis), degrees of freedom (df), sum of squares (SS), mean square error (MSE), expected mean square (EMS), F-value, and the probability that the value will be greater than F (p>F).

Source	df	SS	MSE	EMS	F-value	p>F
Locus	8	6.91812	0.864765	5.57934	2.81	0.0117
Population	4	0.70668	0.17667	16.99069	0.47	0.7562
Individual(Population)	38	8.238375	0.216799	9.175745	1.08	0.3474
Locus(Population)	32	11.47759	0.358675	8.12624	1.79	0.0069
Residual	303	60.67225	0.200238	0.2002	---	---

Figure 2.

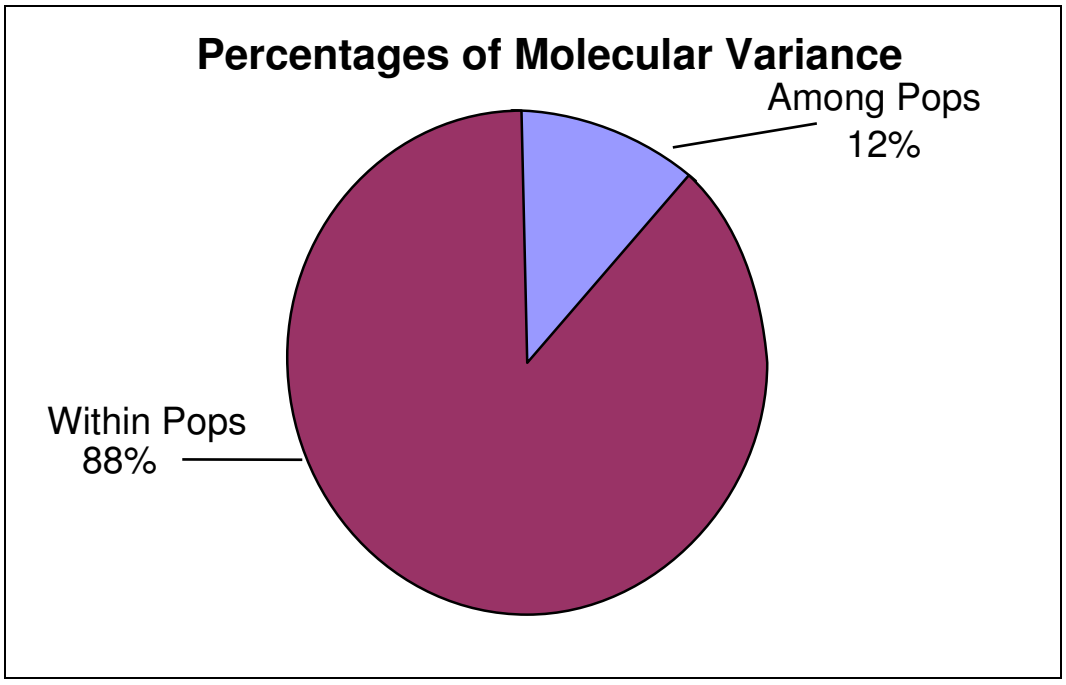

Percentages of Molecular Variance
Among Pops
12%
Within Pops
88%

Table 7. Summary AMOVA table, describing the among and within population calculations (Source), degrees of freedom (df), sum of squares (SS), mean square error (MS), the estimated variance, and percentage. The table also describes the Phi_{PT} value and probability.

Summary AMOVA Table									
Source	**Df**	**SS**	**MS**	**Est. Var.**	**%**		**Stat**	**Value**	**Prob**
Among Pops	4	69.243	17.311	1.134	12%				
Within Pops	38	315.618	8.306	8.306	88%		**PhiPT**	0.120	0.001
Total	42	384.860	25.616	9.440					

Table 8. Data used for the Mantel test described in tables, with panel a) describing the geographic distances between populations, and panel b) describing the genetic distances in the form of Nei's D.

a)

	Geographic Distance using Latitude/Longitude				
St. Croix	Bourbeuse	Saline	Ouachita	Little	
0.000					St. Croix
794.891	0.000				Bourbeuse
1345.499	563.298	0.000			Saline
1306.383	542.427	91.254	0.000		Ouachita
1287.832	584.706	264.535	175.005	0.000	Little

b)

Nei's Genetic Distance via Phylip-GenDist for Adjusted Allele Frequencies					
St. Croix	Bourbeuse	Saline	Ouachita	Little	
0.000					St. Croix
0.195	0.000				Bourbeuse
0.164	0.228	0.000			Saline
0.185	0.187	0.083	0.000		Ouachita
0.265	0.274	0.240	0.222	0.000	Little

Figure 3.

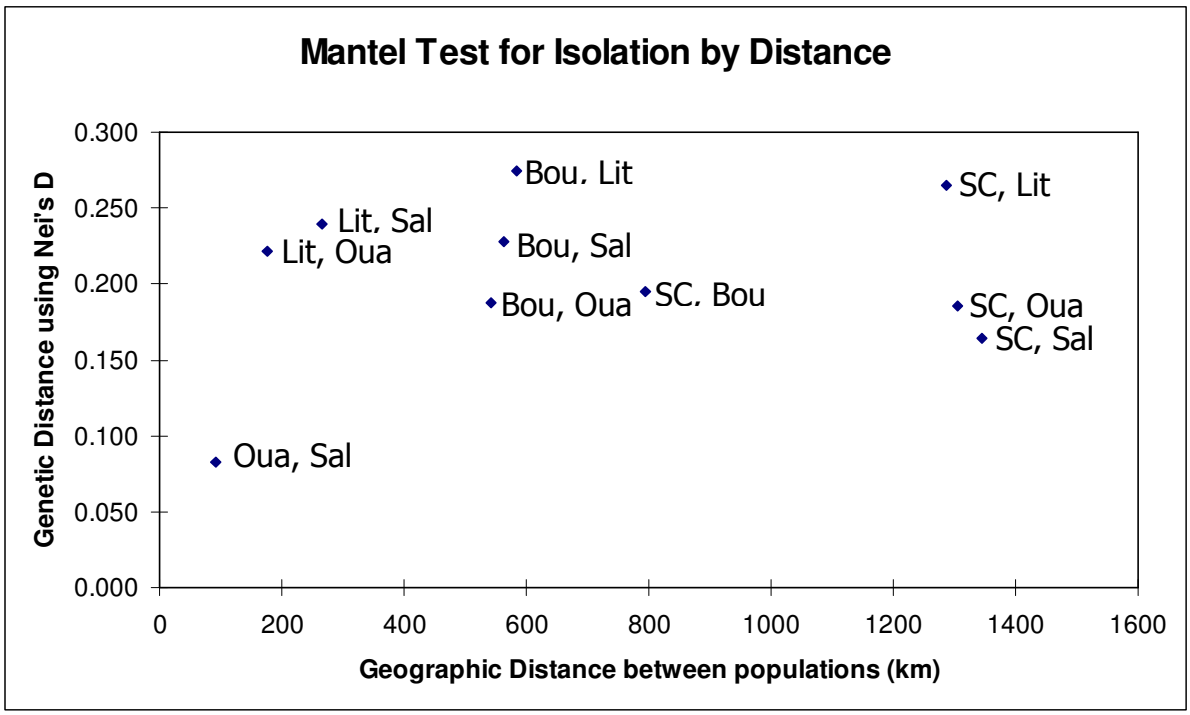
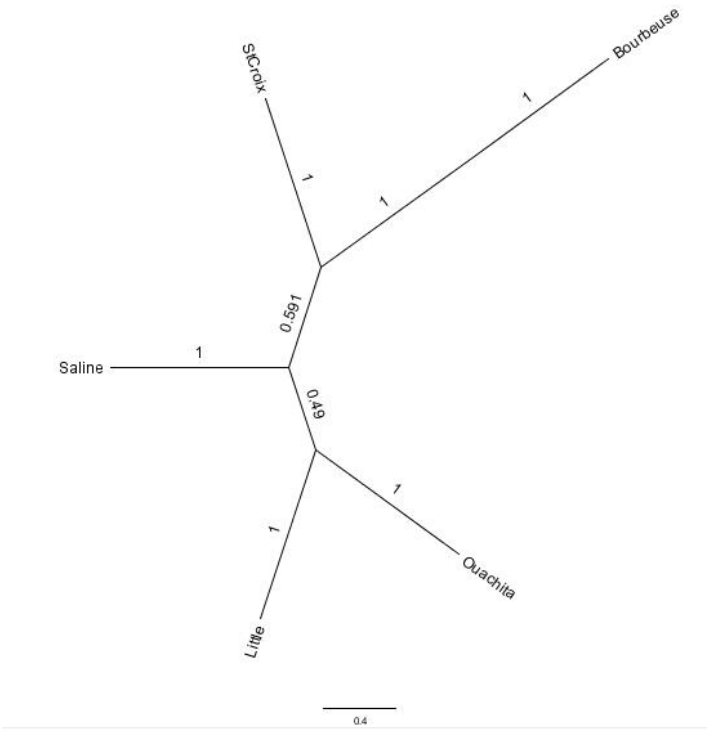

Mantel Test for Isolation by Distance
Genetic Distance using Nei's D
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
200
400
600
800
1000
1200
1400
1600
Geographic Distance between populations (km)
Bou. Lit
SC, Lit
Lit, Sal
Lit, Oua
Bou, Sal
Bou, Oua
SC. Bou
SC, Oua
SC, Sal
Oua, Sal

Figure 4.
a)


StCroix
Bourbeuse
1
1
1
0.591
Saline
1
0.49
1
1
Ouachita
Little
0.4

b)

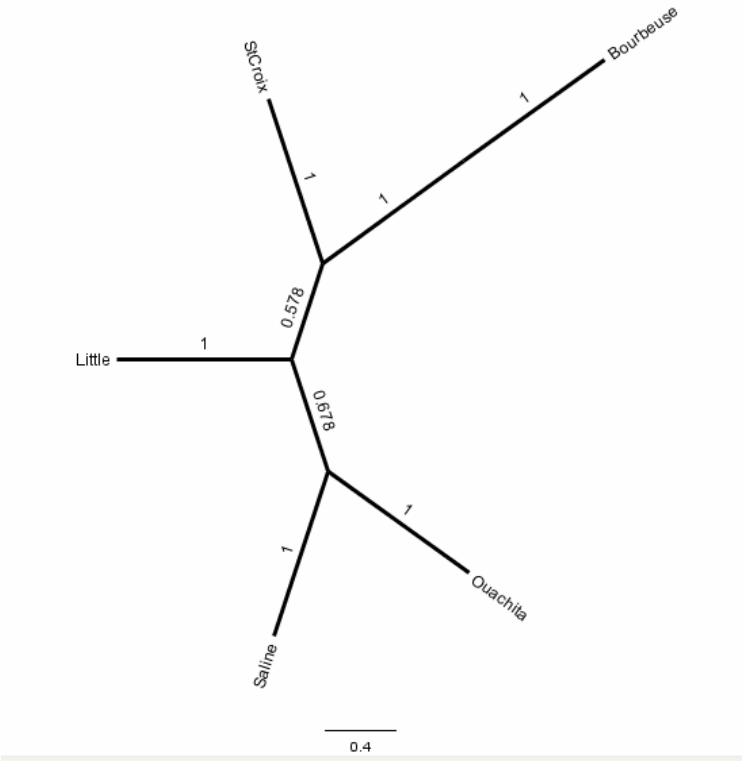

StCroix
Bourbeuse
1
1
1
0.578
Little
1
0.678
1
1
Ouachita
Saline
0.4

Table 9. Pairwise estimates for effective rates of migration based on F_{ST} values using GENETIX (Belkhir, 1999).

N_m for Original Allele Frequencies using F_{st}		
Population 1	**Population 2**	**Nm**
St. Croix (n=13)	Bourbeuse (n=3)	3.85
St. Croix (n=13)	Saline (n=14)	2.57
St. Croix (n=13)	Ouachita (n=10)	1.71
St. Croix (n=13)	Little (n=3)	1.41
Bourbeuse (n=3)	Saline (n=14)	3.94
Bourbeuse (n=3)	Ouachita (n=10)	9.64
Bourbeuse (n=3)	Little (n=3)	1.70
Saline (n=14)	Ouachita (n=10)	4.38
Saline (n=14)	Little (n=3)	2.40
Ouachita (n=10)	Little (n=3)	2.70
	Average	**3.43**

Table 10. Description of average, median, and lower and upper 95% confidence intervals for the effective population size (N_e) for each population of *Quadrula fragosa.*

Population	Mean	Median	Lower 95% CI	Upper 95% CI
St. Croix, Minnesota/Wisconsin	22.0649	22.14552	17.22313	34.29765
Bourbeuse, Missouri	----------Too much missing data-Test not performed----------			
Saline, Arkansas	16.28879	16.42933	12.97666	23.9699
Ouachita, Arkansas	16.41563	16.26677	12.6542	25.7874
Little, Oklahoma	3.136874	3.128565	2.562882	4.031075

CHAPTER 5 - CONCLUSION

The goal of the phylogenetic study was to determine the placement of *Quadrula fragosa* within the genus *Quadrula* and determine if it is a separate species from *Quadrula quadrula.* Using phylogenetic methods, we determined that *Quadrula fragosa* is a separate species, based on its formation of a monophyletic clade separate from *Q. quadrula.* This strongly supports the federal endangered species listing by USFWS in 1991. The assignment of specimens found in Missouri, Arkansas, and Oklahoma is also supported by our phylogenetic analyses (maximum parsimony and Bayesian). Although current knowledge of the *Q. fragosa* range has been limited to a single population in the northern St. Croix River between Minnesota and Wisconsin, this study shows that the range of *Q. fragosa* is more extensive than described at its listing. With the number of people in the field monitoring mussel communities, it is possible that new populations may be discovered in adjacent river systems. These results are vital to the United States Fish and Wildlife Service to resolve the disagreement in the ambiguity in morphology between the two species. Without this phylogenetic study, the USFWS would be unable to proceed with proper and effective conservation plans because the population structure of this endangered mussel would remain unknown. Results from this study are also important for considerations for reclassification to threatened and delisting of *Q. fragosa.* For example, the first criterion for reclassification in the USFWS Recovery Plan states that there must be three discrete populations in at least two tributaries of the Mississippi River drainage (Vaughan, 1997). This study concluded that there are at least five populations of *Q. fragosa* that exhibit a high degree of differentiation with the majority of genetic variation found within each population. Further work needs to

be performed to assess the reproductive viability and population longevity and persistence of these distinct populations.

This study also sought to examine how genetic diversity is distributed across *Q. fragosa,* and how much is found within or between the known five populations using microsatellite markers. Results from this study indicate that the populations exhibit a high degree of genetic distance, little to no gene flow between populations, and that most of the genetic variation of the species is found within each *Q. fragosa* population. Since mussels are sessile and populations of *Q. fragosa* are separated by hundreds of river miles, high gene flow was not expected, and one would expect to find more variation occurring within the populations instead of between them. This hypothesis was supported with the AMOVA results. Knowing that a higher degree of genetic variation is found within populations, the amount of genetic differentiation, as calculated using F_{ST}, should increase as populations are more differentiated from each other. Indeed, this was found with the Saline and Ouachita populations, and with the St. Croix and Little populations. The St. Croix River between Minnesota and Wisconsin is a tributary to the upper Mississippi River that flows into the Gulf of Mexico, but the Little River in Oklahoma is a tributary to the Red River which flows through Arkansas and part of Louisiana before joining the Mississippi River. Therefore, the two populations do not have a way to exchange alleles downstream of each other, increasing the amount of differentiation between them. High rates of inbreeding were found in all populations, but not at all loci within each population. This might be explained by the fact that mussels live in dense mussel beds. Even though the mussels use a fish host to complete glochidial metamorphosis and dispersal, if the fish does not move far due to river alterations, or if multiple juvenile mussels drop off the fish at the same time, it is possible for siblings to

land next to each other. When reproduction occurs the next year, a male could fertilize his sibling simply because she was the nearest female that took up sperm.

When considering species rehabilitation, it is important to consider the number of breeding individuals, N_e, within each population. This is important because a population can be large, but if there are few breeding individuals, then rates of inbreeding and genetic drift can increase and cause population decline. Even though, ONeSAMP (Tallmon, 1998) provided estimates for effective population size, one must hesitate before accepting these numbers at face value. Since *Q. fragosa* is an endangered species, it is difficult to obtain an accurate representation of each population because (as the nature of working with endangered species) it is difficult or impossible to have large sample sizes. A small effective population size does not necessarily mean that a new population can be started with that amount of individuals because they may not contain enough genetic variation to continue the new population. To our knowledge there are no other reports of estimated effective population sizes using ONeSAMP (Tallmon, 2008) for other freshwater mussel species, making this a stepping-stone for future work.

Though this study has uncovered significant information about the phylogenetic placement and population dynamics of *Quadrula fragosa,* there is still much work to be done. Since *Q. fragosa* requires a fish host to complete its juvenile development, population dynamics, reproductive/migratory behaviors, and life history traits must be more deeply explored. Having a firm grasp of the early development of glochidia and their interactions with the host fish will be a valuable component to their conservation and restoration.

This project will also serve as an example of the development and implementation of microsatellite markers for freshwater mussels. With little knowledge about the system and

the rapid decrease of freshwater mussels in the United States, a method to quickly assess the conservation status of threatened and endangered species will be important for their conservation. The techniques, tests, and results presented in this thesis may be of valuable insight to mussel and conservation biologists. It provides techniques and data to other scientists studying microsatellites and/or endangered species. The applications of tests provided an insight to the species with a small sample size, only 43 individuals from five populations.

This study suggests that translocation programs involving *Q. fragosa* should not be implemented in the near future. Far too little is known about the habitat specifics (proper substrate, water conditions, and fish host present) and life history traits to securely begin new populations in the historical distribution of *Q. fragosa.* The possibility of local adaptations of *Q. fragosa* populations needs to be considered as well. Since the five populations have a high degree of genetic differentiation, it is possible that the northern St. Croix population has adapted to colder water temperatures while the Saline, Ouachita, and Little populations have adapted to warmer water temperatures. The difference on water temperatures would have an effect on juvenile mussel development, and possibly affect the reproductive periods of adult mussels. Steingraeber *et al.* (2007) determined that *Q. fragosa* juveniles develop at a faster rate in warmer waters than in cooler waters. Before translocating individuals, one must also consider how the removal of individuals will affect the donor population, the recipient population, and/or the augmentation of a new population. Will removing individuals cause in increased rate of inbreeding due to the removal of variable alleles? Will the recipient population experience outbreeding depression, washing out any local variation the population

may have? Will a new population experience an increased level of inbreeding due to the founder effect?

A more feasible option is the implementation of captive breeding programs. If mussels could be reared in an artificial, yet similar, lab environment, offspring could be introduced back into the population to increase the size, or could be introduced into other populations in hopes of increasing the amount of genetic diversity. Like translocation programs, though, one must be wary of creating an outbreeding depression in the augmented population. If a captive reared population were to become robust enough, they could begin to be introduced into the historical distributions, provided that the habitat is once again suitable for the species.

The ultimate goal for any endangered species, including *Q. fragosa,* is the reclassification to threatened, and eventually delisting the species. In addition to three discrete populations in at least two tributaries of the Mississippi River, other criteria for reclassification include three viable populations (using recruitment, population size, age and genetic structure), persistence (looking at longevity and population surveys), and long-term habitat protection (based on physical, chemical, and biological habitat, and harvest and toxic spill protection) (Vaughan, 1997). As described earlier, this study found that there are at least five discrete populations: St. Croix, Minnesota/Wisconsin; Bourbeuse, Missouri; Saline, Arkansas; Ouachita, Arkansas; and Little, Oklahoma, meeting the first criterion, but I would argue that *Q. fragosa* does not meet all of the criteria for delisting. For example, the next criterion states that the three populations must be viable with recruitment, have an appropriate population size, age and genetic structure (Vaughan, 1997). Even though this study found five populations, I would argue that the Bourbeuse River population in Missouri,

is no longer reproducing and cannot be considered a viable population because only three individuals have been found in the past four years. Also, too little is known about the size and genetic structure of each population to reach a conclusion to address the criteria. The third criterion is that all three populations are persistent in terms of longevity (remaining for 24 years) and performing population surveys every 5 years (Vaughan, 1997). Again, it is too early to tell and we have too little information to address this. The fourth criterion is that the populations have long-term habitat protection (Vaughan, 1997), and this will clearly take time to execute. Since all of these criteria will take a substantial amount of time to develop, implement, and monitor, reclassifying *Quadrula fragosa* as threatened is inappropriate at this time.

Finally, if a successful rehabilitation program is to take place with *Quadrula fragosa,* the public must be involved in its assessing the success of the population(s), and aware of the dire need to conserve the species. Successful rehabilitation programs include the American bald eagle, the California condor, and the timberwolf. These species were made into icons for preservation, and the public responded by improving habitat, decreasing poaching, repealing use of hazardous chemicals, etc. To prevent *Q. fragosa* from joining the approximate 50% of freshwater bivalves that are already extinct or in danger of becoming extinct (Bogan, 1993), the public must realize that *Q. fragosa* is a special type of freshwater "clam," and that its existence is vital to the health of their waterways and persistence of biodiversity.